From the Department of Microbiology, Tumor and Cell Biology

Karolinska Institutet, Stockholm, Sweden

GERMINAL CENTERS - WHY AND WHY NOT?

THE HUMORAL RESPONSE IN PRIMARY IMMUNODEFICIENCY

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Karolinska Institutet

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Germinal Centers – Why and Why Not?

The Humoral Response in Primary Immunodeficiency

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Till min mormor

"And sometimes… you just have to jump off a bridge
& hope like hell that you learn to fly on the way down"

-Anonymous

ABSTRACT

An efficient and regulated immune system is required for protection against pathogens. B cells are required for a humoral response. For a sustained humoral response against pathogens germinal center (GC) reactions are required. In the GC reaction, B cells with antigen-specific B cell receptors are selected and expanded. The GC contains follicular dendritic cells (FDCs) presenting antigens to the B cells, T follicular helper cells promoting proliferation of cognate B cells and highly proliferating B cells expressing activation induced deaminase (AID) in order to produce high-affinity and isotype-switched antibodies.

In this thesis I aimed to define the unique and redundant functions of two actin-polymerizing proteins, WASp and N-WASp, the importance of the RhoGTPase Cdc42 and the consequences of mutated AID in the GC reaction. In **paper I** we discovered that naïve WASp-deficient (WKO) mice and WASp-deficient mice with N-WASp conditionally deleted only in B cells (cDKO) had increased titers of autoreactive antibodies (IgG and IgM, respectively). Both WKO and cDKO B cells got sufficient help from cognate T cells, while the polarization of GCs and the FDC network was altered upon autoantigen challenge. cDKO mice may have increased activation threshold of the B cell receptor in B cells resulting in reduced autoreactive IgG. In **paper II** we revealed the distinctive and partly redundant function of WASp and N-WASp in B cells. We observed reduced marginal zone cellularity in WKO mice and even more pronounced in cDKO mice when compared with wild type mice. The mild phenotype of reduced GC and humoral response in WKO mice previously reported was markedly defected in cDKO mice after antigen challenge, promoting the notion of overlapping functions of WASp and N-WASp in B cells. In **paper III** we investigated the role of Cdc42, that activates both WASp and N-WASp, in B cells. Mice with deleted Cdc42 conditionally in B cells had reduced marginal zone and follicular B cell numbers and reduced GC B cell number associated with reduced specific antibody titers in serum after antigen challenge. In **paper IV** we identified a mouse model for one of the most common mutations in AID leading to the hyper-IgM syndrome. The replacement mutation in amino acid position 112 resulted in a catalytic dead AID protein with spontaneous GC formation, and lack of high affinity or class-switched antibodies.

Altogether, the work presented in this thesis enlightens the significance of tightly regulated B cells for a sufficient, and not autoreactive, humoral immune response. Specifically, this thesis has contributed to our understanding of the importance of a functional AID protein in B cells. Moreover, this thesis has helped in defining the precise activation of the actin regulating proteins in B cells to form antigen-induced GCs and for production of class-switched antibodies with high affinity for the antigen.

LIST OF SCIENTIFIC PAPERS

I. Carin I.M. Dahlberg, Magda-Liz Torres, Sven H. Petersen, Marisa A.P. Baptista, Marton Keszei, Stefano Volpi, Emilie K. Grasset, Mikael C.I. Karlsson, Jolan E. Walter, Scott B. Snapper, Luigi D. Notarangelo, Lisa S. Westerberg.
Deletion of WASp and N-WASp in B cells cripples the germinal center response and results in production of IgM autoantibodies
Submitted

II. Lisa S. Westerberg, Carin Dahlberg, Marisa Baptista, Christopher J. Moran, Cynthia Detre, Marton Keszei, Michelle A. Eston, Frederick W. Alt, Cox Terhorst, Luigi D. Notarangelo, Scott B. Snapper.
Wiskott-Aldrich syndrome protein (WASP) and N-WASP are critical for peripheral B-cell development and function
Blood. 2012 Apr 26;119(17):3966-74

III. Natalija Gerasimicik, Carin I.M. Dahlberg, Marisa A.P. Baptista, Lisa S. Westerberg, Eva Severinson.
B cells devoid of the Rho GTPase Cdc42 are severely deficient in T cell dependent signalling
Submitted

IV. Carin I. M. Dahlberg*, Minghui He*, Torkild Visnes, Magda Liz Torres, Elena M. Cortizas, Ramiro E. Verdun, Lisa S. Westerberg, Eva Severinson, Lena Ström.
A Novel Mouse Model for the Hyper-IgM syndrome: A Spontaneous AID Mutation leading to Complete Loss of Immunoglobulin Class Switching and reduced somatic hypermutation
*J Immunol. 2014 Nov 1;193(9):4732-8 *Equal contribution.*

RELATED PUBLICATIONS OUTSIDE THE THESIS

N-wasp is essential for the negative regulation of B cell receptor signaling
PLoS Biol. 2013 Nov;11(11):e1001704. Chaohong Liu, Xiaoming Bai, Junfeng Wu, Shruti Sharma, Arpita Upadhyaya, Carin I. M. Dahlberg, Lisa S. Westerberg, Scott B. Snapper, Xiaodong Zhao, Wenxia Song.

B cell-intrinsic deficiency of the Wiskott-Aldrich syndrome protein (WASp) causes severe abnormalities of the peripheral B-cell compartment in mice
Blood. 2012 Mar 22;119(12):2819-28. Mike Recher, Siobhan O. Burns, Miguel A. de la Fuente, Stefano Volpi, Carin Dahlberg, Jolan E. Walter, Kristin Moffitt, Divij Mathew, Nadine Honke, Philipp A. Lang, Laura Patrizi, Hervé Falet, Marton Keszei, Masayuki Mizui, Eva Csizmadia, Fabio Candotti, Kari Nadeau, Gerben Bouma, Ottavia M. Delmonte, Francesco Frugoni, Angela B. Ferraz Fomin, David Buchbinder, Emma Maria Lundequist, Michel J. Massaad, George C. Tsokos, John Hartwig, John Manis, Cox Terhorst, Raif S. Geha, Scott Snapper, Karl S. Lang, Richard Malley, Lisa Westerberg, Adrian J. Thrasher, and Luigi D. Notarangelo.

CONTENTS

LIST OF ABBREVIATIONS

AID	Activation induced cytidine deaminase
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
Arp2/3	Actin-related protein 2 and 3
BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2
BAFF	B cell-activating factor of the TNF family
BAFFR	BAFF receptor
Bcl-6	B-cell lymphoma 6
BCMA	B cell maturations antigen
BCR	B cell receptor
Blimp- 1	B lymphocyte- induced maturation protein 1
BP-1	Beta protein 1
BrdU	Bromodeoxyuridine
Btk	Bruton's tyrosine kinase
CCL	CC-chemokine ligand
CD	Cluster of differentiation
Cdc42	Cell division control protein 42
CdLS	Cornelia de Lange syndrome
CDR	Complementarity-determining regions
CFSE	Carboxyfluorescein succinimidyl ester
CIP4	Cdc42-interacting protein 4
CLP	Common lymphoid precursor
CLR	C-type lectin receptor
CpG	Cytosine-phosphate-guanosine
CSR	Class switch recombination
CXCL	CXC motif chemokine ligand
CXCR	CXC motif chemokine receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
Dll-1	Deltalike 1
DNA	Deoxyribonucleic acid
DOCK8	Dedicator of cytokinesis 8
DSB	Double-strand break
EBF1	Early B cell factor 1
Ebi2	Epstein-Barr virus induced gene 2
EdU	5-ethynyl-2'-deoxyuridine
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immmunospot
FDC	Follicular dendritic cell
GC	Germinal center
GEF	Guanine exchange factor
HEL	Hen egg lysozyme
HIGM	Hyper-IgM
HSA	Heat stable antigen
HSC	Hematopoietic stem cell
IC	Immune complex
ICAM	Intercellular adhesion molecule 1
ICOS	Inducible T-cell costimulator
IFNγ	Interferon-γ

Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
IRF4	Interferon regulatory factor 4
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
LFA-1	Lymphocyte function associated antigen 1
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTi	Lymphoid tissue inducer
MARCO	Macrophage associated receptor with collagenous structure
MHC	Major histocompatibility complex
MLP	Multilineage progenitors
MTOC	Microtubule organizing center
MZ	Marginal zone
MZM	Marginal zone macrophage
NIPBL	Nipped-B like
NK	Natural killer
NKT	Natural killer T
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
NP-OVA	4-hydroxy-3-nitrophenyl acetyl-ovalbumin
NSG	Next generation sequencing
N-WASp	Neural Wiskott-Aldrich syndrome protein
PALS	Periarteriolar lymphoid sheath
PAMP	Pathogen-associated molecular pattern
Pax5	Paired box protein 5
PC	Phosphorylcholine
PCR	Polymerase chain reaction
PD1	Programmed cell death-1
PRR	Pattern recognition receptor
PS	Phosphatidylserine
Rac	Ras-related C3 botulinum toxin substrate
RAG	Recombination activating gene
RIG	Retinoic acid-inducible gene
RLR	RIG-like receptor
S1P	Sphingosine 1-phosphate
SAP	SLAM-associated protein
SHIP	Src homology 2 (SH2) domain-containing inositol phosphatase
SHM	Somatic hypermutation
SIGN-R1	Specific intercellular adhesion molecule-3-grabbing nonintegrin-related 1
SLAM	Signaling lymphocytic activation molecule
SLC	Surrogate light chain
SLE	Systemic lupus erythematosus
SRBC	Sheep red blood cell
SSB	single-strand breaks
SYK	Spleen tyrosine kinase
TACI	Transmembrane activator and calcium-modulator and cyclophilin ligand interactor
TCR	T cell receptor

TD	Thymus dependent
T_{FH}	Follicular T helper
TGF β	Transforming growth factor-β
TI	Thymus independent
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TNP-KLH	Trinitrophenyl keyhole limpet hemocyanin
T_{reg}	T regulatory
UNG	Uracil DNA glycosylase
VCAM1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen 4
WAS	Wiskott-Aldrich syndrome
WASp	Wiskott-Aldrich syndrome protein
XBP-1	X-box binding protein 1

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system is important for protection against foreign pathogens like virus, bacteria and other microbes. The first lines of defense against infections are the physical and chemical barriers that stop pathogens from entering the body. These barriers include the skin, hair, mucous membranes and production of antimicrobial substances. The second line of defense, the immune system, is activated when the pathogen has reached internal tissues and an immune response is initiated.

1.1.1 The innate immune system

Classically, all immunological reactions have been divided into two branches, the innate and the adaptive immune system, depending on if the receptor recognizing pathogen is encoded in the germline DNA or if gene rearrangement is required[1,2]. Innate immunity represents the first response in an immune reaction and this response is fast and has no ability to form efficient memory responses. The innate immune response recognizes a foreign pathogen by two strategies. First, by identifying markers that are characteristic to the pathogen and not present in the host. Second, by recognition of "missing-self", where the pathogen lacks expression of markers normally expressed by host cells[3,4].

Cells constituting the innate immune repertoire include monocytes, macrophages, dendritic cells (DCs), innate lymphoid cells (ILCs, which includes natural killer (NK) cells) and granulocytes (eosinophils, basophils, neutrophils, mast cells). These cells either express **pattern recognition receptors** (PRRs) or they are recruited to the site of interest by cells expressing PRRs. This family of receptors identify pathogens through recognition of pathogen-associated molecular patterns (PAMPs)[5]. The PRR-family includes several receptors such as **Toll-like receptors** (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and scavenger receptors[6,7]. The TLRs, CLRs and the scavenger receptors are transmembrane proteins while the NLRs and RLRs are located in the cytosol and bind intracellular PAMPs. PRRs also recognize endogenous molecules – damage-associated molecular patterns (DAMPs), released from host cells as a result of necrosis, damage and pathogen infection[6]. The TLRs can be expressed either on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6) or in the lumen of intracellular vesicles (TLR3, TLR7, TLR8, TLR9, TLR11, TLR13) such as endoplasmic reticulum, endosomes, lysosomes and endolysosomes [8]. The intracellular localization of TLRs enables recognition of nucleic acids from viruses and other pathogens in the cytosol of infected cells, thus providing important protection against infectious agents. However, intracellular TLRs entail the risk of unwanted

immune responses directed against the host's own nucleic acids and therefore proper localization and trafficking of TLRs is required.

In addition to the two main groups of innate and adaptive leukocytes, there are two groups of cells that fit into neither. The first group is the ILCs. As the name implies these cells are derived from the common lymphoid precursors (CLPs), which normally are considered precursors of cells belonging to the adaptive immunity, with exclusion of NK cells - which are a part of the ILCs. Three main features define ILCs: their antigen receptor is encoded in the germline DNA and does not require rearrangement by **recombination activating gene (RAG)**; they lack myeloid cell and DC phenotypically markers; and they have a lymphoid morphology[9,10].

The second group of cells includes four cell populations; B1 B cells, marginal zone (MZ) B cells, γδ T cells and natural killer T (NKT) cells[11-13]. These populations form a part of the adaptive immune system but are characteristically different from the conventional lymphocyte cells. They all have B cell receptors (BCRs) or T cell receptors (TCRs) that require RAG-dependent rearrangement, but the antigen receptors are poorly diversified. The B1 B cells are dominant in the peritoneal cavity but also found in the spleen, where the MZ B cells are located. These two innate-like B cell populations produce **natural antibodies**[14], a pool of antibodies occurring without previous antigen challenge. Natural antibodies are primarily immunoglobulin M (IgM) but also include IgG and IgA. Their characteristic features are polyreactivity, low affinity for the antigen, and a high frequency of autoreactivity[15,16]. Natural antibodies can be considered a part of the innate immune system since they are involved in neutralization of pathogens, activation of the complement cascade and clearance of apoptotic cells[16,17]. Activation-induced cytidine deaminase (AID)-deficient mice lack IgG antibodies and they have an increased risk of infections. By passive immunization with IgG antibodies, the AID-deficient mice are protected against bacterial infection indicating that natural antibodies are a part of the innate immune system[18].

1.1.2 The adaptive immune system

Compared to the innate immune system the adaptive immunity is more complex and the response peaks usually 7–14 days after recognition of a foreign pathogen. The response is slow because the antigen has to be taken up and processed by antigen presenting cells (APCs) and introduced to the T (thymus-derived) and B (bursa- or bone-marrow-derived) lymphocytes representing the adaptive immunity. The hallmark of the adaptive immune response is memory; it remembers pathogens from previous encounters. Immunological memory enables the immune system to mount a rapid secondary response upon re-exposure to the pathogen. However, the possibility to remember antigens together with an effective immune response involving many cell types with the ability to mutate and change their

antigen-recognition receptor make the adaptive immune system a risk factor in terms of autoimmunity[2].

1.1.3 B cell development - from bone marrow to spleen

In the early 1970s when the first expansive review of the T and B lymphocytes were published, the site of B cell development in mammals was still not clear[19]. Since then, these two cell types of the adaptive immune system have been extensively studied.

Today it is know that conventional B cells, or B2 B cells, are derived from hematopoietic stem cells (HSCs) in primary lymphoid organs (fetal liver and bone marrow after birth) and migrate to secondary lymphoid organs (mainly spleen and lymph nodes) during development. The B cell development described in this section is focused mainly on B2 B cells. The development of B1 B cells is debated and less understood. B1 B cells, mentioned in the innate immune system section, most probably have another maturation pathway that is different from B2 B cells. Some distinct differences between the B cell subsets are that B1 B cells have intermediate rather than high B220 expression, cluster of differentiation (CD) $CD11b^+$ (Mac-1, in peritoneal cavity), $CD5^+$ (B1a), natural antibody production and ability of self-renewal. Transplantation studies with adult bone marrow or neonatal liver show that adult bone marrow does repopulate the B2 B cells while the B1 cell population is less satisfactory repopulated. However, transplantation of neonatal liver cells repopulate both populations efficiently, suggesting that B1 and B2 cells develop separately[20]. During embryonic development the bone marrow is populated by stem cells originating from fetal liver. Both B1 and B2 B cells develop from HSCs to CLPs through multilineage progenitors (MLPs). The CLP subset can only give rise to lymphocytes (i.e. T, B and NK cells) and is directly prior to B linage commitment where the cells become B lineage restricted and express B220[21].

A key regulator of early B cell development is interleukin-7 (IL-7) produced by stromal cells in the fetal liver, bone marrow, thymus and spleen. The IL-7 receptor α chain is upregulated on CLP cells. IL-7 or IL-7 receptor-deficient mice have a severe block in early B cell development[22,23]. The B cells that are found in the periphery of these mice are of MZ and B1 B cell phenotype[24]. These cells originate from the fetal liver where IL-7 has shown not to be absolutely required for B cell development[25,26]. In contrast to murine B2 lymphocytes development it has been shown that human B cell generation is largely or entirely IL-7-independent. Comparable to the mouse data, B cells produced in patients with a defective IL-7 receptor α subunit expression come from fetal or neonatal development, although the exact origin and development are not yet fully understood[26,27].

After lineage commitment the B cell development can be divided into several maturation stages. There are three different nomenclatures of early B lineage cells; Basel nomenclature, Philadelphia nomenclature and the “the Hardy fractions” (described 1991 by Richard Hardy

and colleagues) (Table 1)[28,29]. Since the Hardy classification and the Philadelphia nomenclature are used in the papers included in this thesis, I will mainly refer to the Hardy classification with the Philadelphia nomenclature in parenthesis.

Hardy classification	**Fraction A**	**Fraction B/C**	**Fraction C´**	**Fraction D**	**Fraction E**
Philadelphia nomenclature	Pre-pro B cell	Pro B cell	Early pre B cell	Late pre B cell	Immature B cell
Basel nomenclature	Pro B cell	Pre B-I cell	Pre B-I cell	Pre B-II cell	

Table 1. Nomenclature of B cells in the bone marrow[28].

The first stage, fraction A (pre-pro B cell), can be recognized by expression of $CD43^+$, $B220^+$ and absence of CD19 and CD24 (heat stable antigen, HSA) expression [30]. Specificity for the B cell linage comes from activation of the helix–loop–helix protein E2A and the early B cell factor 1 (EBF1) which activate B-lymphoid genes, such as Igα and Igβ which are important for intracellular signaling of the BCR later on. Mice with either of these transcriptions factors mutated have a B cell arrest prior fraction A[31]. Fraction A has little, if any, gene rearrangement of the BCR[32]. Sequential gene rearrangement of the antigen receptor is found first in the next stage, fraction B/C (pro B cell) that expresses all four markers $CD43^+$, $B220^+$, $CD19^+$, $CD24^+$ but still does not express a pre-BCR or BCR on the cell surface[29]. B220 and CD19 are two markers commonly used to characterize mouse B cells in the periphery.

The antigen receptor gene rearrangement is called **V(D)J recombination**, and induces recombination of the variable (V), diversity (D) and joining (J) segments. V(D)J segment recombination requires the expression of the proteins RAG1 and RAG2[33,34]. Two factors controlling the V to DJ rearrangement are IL-7 and the transcription factor paired box protein 5 (Pax5)[35,36]. If successful rearrangement of the immunoglobulin heavy (IgH) chain locus occurs the cell goes into fraction C´ (early pre B cell). This fraction is the last to express CD43 – cells in transition from pro-B to pre-B. The fractions A, B, C and C´ can be separated by their surface expression of CD24 and beta protein 1 (BP-1). Fraction C´ is the first fraction to express the pre-BCR on the cell surface.

The **pre-BCR** is important for testing the potential binding capacity of the IgH chain, called positive selection. If the IgH chain is autoreactive it may undergo additional rounds of rearrangement to get a functional IgH chain. The pre-BCR is formed by the rearranged IgH

(Igμ) and the Ig light-(IgL) chain-like structure; the surrogate light chain (SLC). The SLC consists of two proteins: VpreB and λ5. SLC-deficient mice have impaired B cell development after fraction B/C (pro B cell) and have increased levels of autoreactive IgM antibodies produced by MZ B cells[37-39]. In the pre-BCR complex there is also the transmembrane signaling proteins, Igα (CD79a) and Igβ (CD79b). The enzyme spleen tyrosine kinase (SYK) can phosphorylate tyrosine residues on Igα's and Igβ's cytoplasmic domains, immunoreceptor tyrosine-based activation motif (ITAM), and signaling molecules such as Bruton's tyrosine kinase (Btk) can bind to the phosphorylated structure and promote pre-BCR and BCR signaling by stimulating Ca^{2+} flux[40]. The BCR is built up by four components, two IgH chains and two IgL chains, which consist of either Igκ or Igλ (only in 5% of murine B cells)[41]. Like the pre-BCR, the BCR has Igα and Igβ associated to it. V(D)J rearrangement of the IgH chain occurs in fraction B/C while for IgL locus it occurs in fraction D (late pre-B cell). At this stage, 5×10^{13} different antibody specificities recognizing antigen epitopes could theoretically be formed, depending on the IgH chain variation. Many of the formed clones will be excluded later on because of unwanted affinities, for example too strong reactivity to autoantigens[42]. RAG-expression is tightly regulated and almost exclusively expressed during this part of B cell maturation. However, there is one B cell population which develops in the lamina propria and undergoes RAG-dependent V(D)J recombination in the mouse intestine[43]. Another exception is a GC B cell subset that are able to turn on the expression of λ5 and RAG1 and RAG2, to make V(D)J recombination possible in the periphery[44]. However, Gärtner et al. together with other groups suggest that these RAG-positive B cells in the periphery actually are progenitor/precursor B cells from the bone marrow accumulated in the spleen[45].

In the progression from fraction D to fraction E (immature B cell) the B cell's newly formed BCR is tested for potential autoreactivity in order to eliminate autoreactive B cells in the central tolerance checkpoint. This negative selection results either in deletion of the cell or new recombination of the already existing BCR. Receptor editing involves mainly the IgL chain since the IgH chain is tested during the pre-BCR process. Receptor editing includes re-expression of RAG proteins so that new gene rearrangement can occur[46]. Productive rearrangement of both IgH and IgL chains in pre-B cells which induces BCR signaling, results in immature IgM^+ B cells that can leave the bone marrow and migrate to the spleen and other secondary lymphoid organs to take part in humoral immune responses[28]. Of the $1\text{-}2 \times 10^7$ IgM^+ immature B cells developed every day only 10% leaves the bone marrow as transitional B cells[47].

So far, three subsets of **transitional B cells** have been identified and defined by their surface phenotype (in mouse; T1, T2 and T3). The two first subsets were found by using the surface markers for B220, CD23 (FcεRII), CD21 (complement receptor 2) and IgM. T1 cells can be separated from T2 cells by lack of IgD, CD23 and CD21 protein expression[48]. In 2001 Allman and colleagues characterized the third population first by positive selection on $B220^+CD93^+$ (AA4.1) cells and then separating the population by IgM and CD23

expression[49]. More recent studies on the T3 subset in mice suggest that this population might represent anergic cells that that will not progress into mature B cells[50,51].

Transitional B cells are short lived and only one third of the transitional B cells become mature B cells. One critical selection factor for the B cell to become a long-lived mature peripheral B cell is the BCR signaling. In humans around 75% of the early immature B cells in the bone marrow recognize autoantigens. This number is reduced to ~40% when the cells are ready to leave the marrow, and in the pool of mature circulating B cells the percentage of autoreactive B cells is ~20%. The first elimination of autoreactive B cells is during the central tolerance checkpoint and the second occurs outside the bone marrow and is called the peripheral tolerance checkpoint in the T2 stage. This checkpoint probably occurs when the BCR recognizes new autoantigens that are not present in the bone marrow and when B cell-activating factor of the TNF family (BAFF) receptor (BAFFR) is expressed[52,53].

Two cytokines that have a profound impact on B cell survival and development are, BAFF and a proliferation-inducing ligand (APRIL), both members of the TNF family. BAFF and APRIL are both produced by stromal cells in the splenic red pulp and provide basic survival signals to B cells[54]. Even though stromal cells are capable of producing BAFF, this cytokine is primarily produced by follicular dendritic cells (FDCs) in the B cell follicles of the spleen and the lymph nodes, and can also be produced by other immune cells, such as macrophages, DCs and neutrophils[55,56]. Both APRIL and BAFF interact with the receptors transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) and B cell maturations antigen (BCMA), which are present on B cells and BAFF also interacts with the BAFF receptor[57]. BCMA expression is restricted to plasmablasts and plasma cells, and promotes the survival of long-lived plasma cells.

To maintain the homeostasis of mature peripheral B cells at least two receptors, BAFFR and BCR, are required to signal. BAFF receptor signaling is required for all B cell populations after the T1 stage, except for memory B cells and B1 B cells. BAFF or BAFFR-deficient mice have a marked reduction in B2 B cells while the B1 B cell population in peritoneum stays unaffected[58,59]. Mice deficient in BAFF or BAFFR are able to form GCs efficiently, but they are not able to sustain the GC reaction[60]. Overexpression of BAFF leads to increased size of the B cell compartment and also survival of autoreactive clones, which would otherwise have been deleted in the periphery[61]. Similarly, patients with autoreactive B cells and autoimmune disorders have been correlated with increased serum titers of BAFF. Interestingly, autoreactive B cells that are induced by overexpression of BAFF do not require T cell help [62].

There are two major driving forces for the B cell to become either a follicular B cell or a MZ B cell. First off is the signaling through the BCR. Strong BCR signaling favors a follicular B cell development through activation of the Btk pathway, and cells with weaker BCR signaling become MZ B cells. The second important factor for MZ B cell differentiation is Notch signaling[63]. B cells can only become MZ B cells if they receive signals from Notch.

The ligand for Notch2 is deltalike-1 (Dll-1), expressed by endothelial cells in the red pulp of the spleen[64]. Strong BCR signaling leads to activation of Btk, which inhibits Notch signaling and reduces the MZ B cell potential. On the other hand, weak BCR signaling leads to Notch2 signaling which in turn activates the transcription factors Mastermind-like 1 (MAML1) and recombining binding protein suppressor of hairless (RBP-Jκ) both of which are essential for Notch signaling. MZ B cell development is defective in Notch2, Dll-1, MAML1 or RBP-Jκ-deficient mice. Reduced expression of the transcription factor E2A and increased expression of the opposing transcriptional factors inhibitor of DNA (Id2) and Id3 levels facilitates MZ B cell development and reduces follicular B cell development. Id2 and Id3 can diminish the function of E2A[63]. Recent data has provided a link between Notch signaling and Id2, since Notch signaling elevates the expression of Id2 in MZ B cells[65].

1.1.4 The spleen

The spleen is the largest secondary lymphoid organ and the site of the adaptive immune response initiation. As described in the previous section one important function of the spleen is the peripheral development of B lymphocytes. The spleen can be subdivided into the red pulp, containing plenty of red blood cells, hence the name, and white pulp where the lymphocytes are located (Fig. 1).

The red pulp is important for filtering the blood and holding numerous macrophages that phagocytose ageing erythrocytes and blood-borne pathogens. It also accommodates plasmablasts and plasma cells that have left the white pulp. Cells can leave the white pulp by upregulation of CXC motif chemokine receptor (CXCR) 4, the receptor for CXC motif chemokine ligand (CXCL) 12 that is expressed by stromal cells, macrophages, neutrophils and DCs in the red pulp[66].

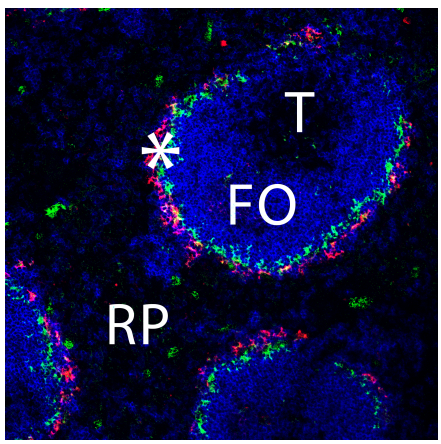

Figure 1. B cell follicle with metallophilic macrophages and marginal zone B cells. Immunofluorescence of a wild type mouse spleen shows $B220^+$ B cell follicles (blue), $CD169^+$ metallophilic macrophages (green) and $CD1d^+$ marginal zone B cells (red). The red pulp (RP), B cell follicle (FO), marginal zone (*), and T cell zone (T) are indicated.

The white pulp consists of DCs and lymphocytes, further subdivided in B cell follicles and T cell zones, also known as periarteriolar lymphoid sheath (PALS) (Fig. 1). For B cells to home to the follicles in lymphoid organs the CXCL13[67], produced by FDCs and stromal cells, and its receptor CXCR5 are required[68]. T cells and DCs require the CC-chemokine ligand (CCL) 19 and CCL21, produced by stromal cells, to get to the T cell zones[69]. The receptor for both CCL19 and CCL21 is CCR7, and is expressed by B cells, T cells and DCs, which all have impaired migration in CCR7-deficient mice[70] (Fig. 2).

In mice there is an area next to the white pulp, towards marginal zone, called the marginal sinus. The marginal sinus is the end of the smallest arterial branches in the spleen. The marginal zone and the marginal sinus form a boundary between the red and white pulp (Fig. 2).

The major conventional B cell population in the spleen is the follicular B cells that accounts for 80–90%, while the MZ B cells account for 5–10% of all B cells in an adult mouse. MZ B cells are strategically located in the interface between the circulation and the follicle to remove blood-borne pathogens. Blood, containing the lipid mediator sphingosine 1-phosphate (S1P) flows throw the marginal zone of the spleen. S1P is the only known ligand for the S1P receptor 1 and 3 ($S1P_1$ and $S1P_3$, receptively). The S1P receptors are required for B cells to overcome the CXCL13 mediated attraction to the follicle and exit to the marginal zone. MZ B cells are able to transport antigens to FDCs by shuttling between the marginal zone and the follicle. The shuttling back and forth is regulated by a balanced expression of $S1P_1$ and CXCR5. However, $S1P_1$-deficient B cells are able to localize in the marginal zone of mice lacking CXCL13, the ligand for CXCR5[71,72]. CXCR4 has long time been thought to be the exclusive receptor for CXCL12. Although recent publications have shown that CXCR7 can bind with high affinity to CXCL12 as well and that CXCR7 is upregulated on MZ B cells but not on follicular B cells. A block of CXCR7 leads to reduced number and disrupted architecture of MZ B cell. This implies a role of the chemokine receptor CXCR7 in B cell homing to the marginal zone[73,74].

1.1.4.1 Macrophages in the spleen

In the marginal zone we also find **MZ macrophages** (MZM) that express PRRs such as specific intercellular adhesion molecule-3-grabbing nonintegrin-related 1 (SIGN-R1), scavenger receptor A (SR-A) and macrophage associated receptor with collagenous structure (MARCO) (Fig. 2). SIGN-R1 is a C-type lectin while SR-A and MARCO are both scavenger receptors. MZMs are important for removal of both blood-borne pathogens and of apoptotic cells from the circulation.

Under the marginal sinus, towards the white pulp, is the location of the **metallophilic macrophages** expressing the adhesion molecule CD169 (sialoadhesin). Since it is extremely hard to remove one of the cell populations without influencing the other in experiments, the

specific contribution of MZM and the metallophilic macrophages in the clearance of apoptotic cells is still uncertain. Both cell subsets do express different sets of receptors recognizing apoptotic cells, which makes it possible that they have distinct functions in the removal of dying cells[75] [74].

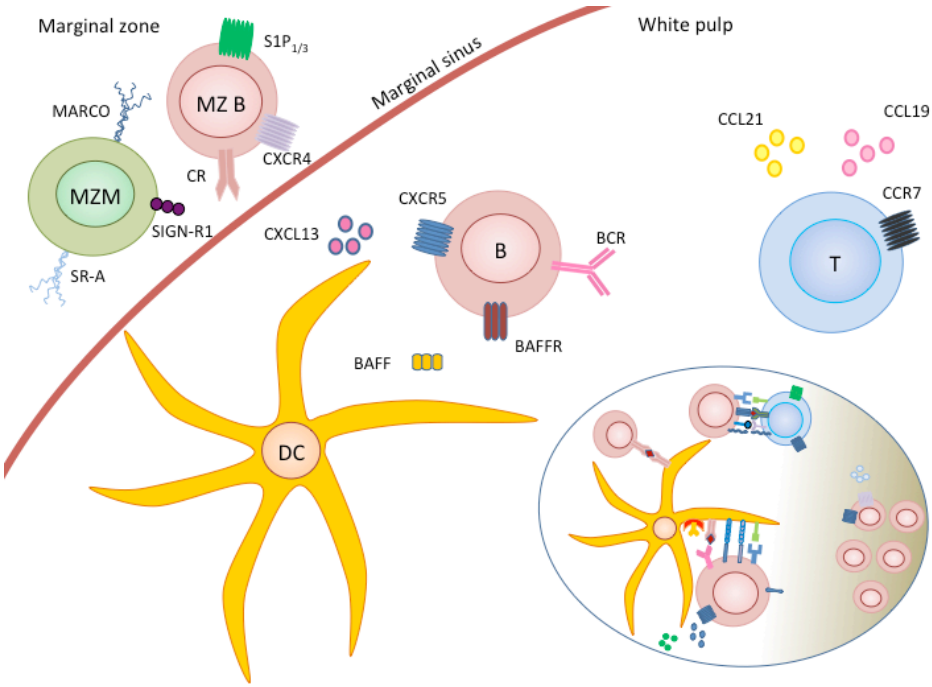

Figure 2. Schematic picture of the splenic white pulp with the marginal zone and the marginal sinus. Important receptors and ligands discussed in the text are highlighted. *Illustration by Emelie Risberg.*

During the germinal center (GC) reaction a large number of B cells go into apoptosis because of failure in antigen recognition. The **tingible body macrophages** are located in the GC to remove all the B cells that go into apoptosis. It is important for the engulfment by the macrophages that the apoptotic cell express phosphatidylserine (PS). PS on the cell surface works as one of several "eat-me" signals, and is normally located cytosolic side of the plasma membrane. PS can be bound to tingible body macrophage by Tim-4 or by the soluble bridging molecules milk fat globule EGF factor 8 protein (MFGE8) and growth-arrest-specific 6 (GAS6), which in turn can be recognized by the phagocyte receptors $\alpha_v\beta_3$-integrin or the tyrosine kinase Mer respectively expressed on the tingible body macrophages. Mer is expressed by red pulp macrophages and DCs but not on MZM or metallophilic macrophages while MFGE8 is only found on tingible body macrophages[76].

There is a small population of macrophages also found in the T cell zone. These macrophages lack markers specific for MZM and the red pulp macrophages, and needs to be more investigated [75].

The marginal zone is populated by three cell types, DCs, MZ B cells and MZMs. DCs can be located in the marginal zone temporarily, but leaves the marginal zone to the T cell zone after activation and antigen uptake[75]. There is close contact between the metallophilic macrophages, MZ B cells and the MZMs, and they influence each other. Several different mouse strains that lack B cell cells do also lack metallophilic macrophages and MZMs[77,78]. Mice lacking CD19, which is only expressed on B cells, have B cells in the periphery but lack MZ B cells[79]. These mice do also lack the $MARCO^{+}SIGN\text{-}R1^{+}$ MZM while the $MARCO^{+}SIGN\text{-}R1^{-}$ cells are still present[80,81]. By depleting $MARCO^{+}$ MZMs with macrophage-depleting liposomes there is a specific reduction of MZ B cells as well. However, *op/op* mice are deficient in metallophilic macrophages while the $MARCO^{+}SIGN\text{-}R1^{-}$ and MZ B cell populations are still present[81,82]. All together we can conclude that to form a proper splenic cell organization the marginal zone cells need to collaborate and influence each other.

The white pulp is restricted for lymphocytes and DCs, though APCs can enter for antigen presentation. There is no blood flow through the white pulp as in the red pulp and large molecules are unable to enter. Small molecules such as chemokines and small antigens are able to penetrate the white pulp by the **conduit system**. A core of collagen fibers and fibroblasts build up the conduit system, which can present transported and locally produced chemokines, CXCL13 in B cell follicle and CCL21 in the T cell zone[83,84].

1.2 THE GERMINAL CENTER

A **germinal center** (GC) is an antigen-driven structure within the white pulp of the spleen. In the GC-process cognate B cells with some affinity for the antigen is selected and high-affinity BCRs are generated by somatic hypermutation (SHM). The final product of the GC is B cells with high affinity for the antigen, which can differentiate into plasma cells or memory cells.

1.2.1 TI and TD immune responses

The division into thymus independent (TI) responses and thymus dependent (TD) responses depends on whether T cell help is required for antibody production or not. The TI response can be further subdivided in two groups depending on the provoking antigen. The TI type 1 group of antigens includes molecules that can bind to the BCR or to the TLRs, exemplified by bacterial lipopolysaccharides (LPS). The TI type 2 group of antigens is characterized by the requirement of clustering and cross-linking of the BCR in order to initiate an immune response. The prime example of a TI type 2 group of antigens is the highly branched polysaccharide Ficoll. TI antigens induce extrafollicular antibody responses and in general do not promote formation of GCs or elicit any memory, even if the primary antibody response can last for a long time[85-87]. TD antigens are processed and presented on major histocompatibility complex (MHC) class II molecules for T cell help in secondary lymphoid organs. TD responses against antigens such as keyhole limpet hemocyanin (KLH) and sheep red blood cells (SRBC) give rise to high-affinity antibody producing cells and memory B cells, conferring long-lasting antibody titers in the serum of challenged individuals. The following sections will focus on TD antigen responses.

1.2.2 The zones

The GC can be divided into two zones, light and dark. They are clearly visual and distinguishable with conventional histology methods. The **light zone** contains many cell populations - DCs (mostly FDCs), B cells (both B cells active in the GC reaction and IgD^+ B cells in transit), T cells (majority is $CD4^+$) and tingible body macrophages that engulf the apoptotic B cells. The **dark zone** is denser and contains almost exclusively B cells (Fig. 3). Initiation of proliferation by GC B cells can occur in the light zone but it is only in the dark zone where the cells can complete the cell-cycle[88,89].

1.2.3 Follicular dendritic cells

Follicular dendritic cells (FDCs) are important for production of affinity maturated B cells. A majority of the recent *in vivo* research in the GC field has been done in lymph nodes. This

is due to the lower optical obstacles in lymph nodes compared to the spleen, enabling the application of new technology such as intravital two-photon microscopy, which relies on the penetration of electromagnetic radiation through tissues. In the lymph node, subcapsular macrophages catch the antigen from the lymph and deliver it to the follicular B cells that are able to transport the antigen to FDCs[90].

The FDCs mature from vascular mural cells, which are cells with contractile function wrapped around the endothelial cells of small blood vessels. FDCs are radiation-resistant which has made the research about FDC development more difficult since donor cells do not repopulate in bone marrow chimeric mice due to the already occupied niche. During the development of lymph nodes, lymphoid tissue organizing cells are triggered by retinoic acid to produce CXCL13 that attracts lymphoid tissue inducer (LTi) cells. LTi cells interact with lymphoid tissue organizing cells and stromal cells through lymphotoxin (LT) and the LT receptor, which together promote lymphocyte and stromal cell accumulation[83]. LTs belong to the tumor necrosis factor (TNF) family, which also includes FasL and CD40L, important for B cells in GCs. For normal generation of FDC structure LTs and TNF produced by B cells are both required. However, for the maintenance of splenic architecture in adult mice, only LTs, and not TNF, are required. Mice deficient in LTα have no lymph nodes or Peyer's patches and fail to form GCs in the spleen in response to injected SRBC. They also fail to form GCs to the other TD antigens (4-hydroxy-3-nitrophenyl) acetyl-ovalbumin (NP-OVA) and trinitrophenyl (TNP)-KLH[91]. Interestingly, dose-dependent affinity maturation towards NP-OVA is found in LTα-deficient mice. LTα-deficient mice injected with 1-5μg NP-OVA show a marked reduction of high-affinity antibodies compared to wild type mice. When instead injected with high dose (200μg) of the same antigen the response depending on SHM and high-affinity antibodies were detectable in both strains, but still no GC formation was detected in the LTα-deficient mice[92]. Blocking the LT-specific LTβ receptor in adult mice leads to severe reduction of B cell follicles, metallophilic macrophages, MZM and no GC formation after SRBC immunization[93].

FDCs produce CXCL13 that attracts B cells and T_{FH} cells to the B cell follicle. As a positive feedback loop, CXCL13 makes B cells produce more LT. The FDC and B cell interaction is facilitated by expression of vascular cell adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1) on the FDCs. The receptors for VCAM1 and ICAM1, very late antigen 4 (VLA-4) and lymphocyte function associated antigen 1 (LFA-1) are expressed on follicular and GC B cells, respectively[94](Fig. 3). FDCs produce several cytokines important for B cell stimulation, such as BAFF and IL-6[95]. IL-6 is known to regulate terminal B cell differentiation. Mice lacking FDCs with capacity to produce IL-6 have reduced GC formation and SHM frequency in GC B cells[96].

The FDCs can retain intact antigen for long periods and they work as a reservoir for antigen during the GC reaction[97]. The FDCs bind the antigen itself or as an immune complex (IC) with antibody and/or with complement, which they trap in recycling endosomal compartments, to protect the antigen from degradation[98]. FDCs with antigen preloaded in IC

are much more potent for B cell activation and antibody production rather than the free antigen[99]. APCs present antigen in the context of MHC molecules. The binding of IC requires the use of different receptors, for example complement receptors 1 and 2 (CR1 and CR2, or CD35 and CD21, respectively) and Fc receptor (FcγRIIB, CD32). FcγRIIB is an inhibitory, low-affinity Fc receptor. In B cells, cross-linkage of the BCR and the FcγRIIB leads to tyrosine phosphorylation of the immunoreceptor tyrosine-based inhibition motif (ITIM). This is followed by upregulation of SH2 domain-containing inositol phosphatase (SHIP) that blocks Ca^{2+} flux due to disassociation of Btk from the plasma membrane, resulting in inactivation of the B cell. FcγRIIB-deficient mice have a reduced capacity to capture IC[100]. Inability to secrete antibodies leads to lack of detectable ICs on FDCs after immunization with NP-conjugated TD antigens. By crossing mice lacking ability to secrete antibodies with transgenic mice with BCR specific for the hapten NP Hannum et al. showed that GC formation and clonal expansion of an antigen-specific B cell are formed without help from FDCs[101]. Mice with a specific deletion of FcγRIIB in B cells have increased antibody titers in general, but also autoreactive antibody clones are increased. This suggests that FcγRIIB has an important role in tolerance regulation both in mature B cells and in the GC response[102]. By expressing high levels of FcγRIIB on the FDCs' membrane, the risk of cross-linkage of BCR and FcγRIIB on the B cell is reduced[103,104]. The FDCs get the IC delivered by noncognate B cells, for example MZ B cells expressing CD21 and CD35 in the spleen. For retention of the antigen by FDCs the CD35 is essential and is expressed in high level on FDCs[98,105]. B cells predominantly express CD21 while FDCs express CD35 to a higher extent. Upon TD antigen challenge the FDCs require CD35 expression to be able to get a strong GC B cell response, while naïve CD35-deficient mice have normal B cell development and antibody titers[106].

1.2.4 T follicular helper cells

Follicular T helper (T_{FH}) cells are associated with GC B cells and are required for normal GC reaction. For the T helper cells to be able to reach the B cells to initiate a GC formation they upregulate CXCR5 and downregulate CCR7 after antigen recognition. To become T_{FH} cells several molecules may play a role: Bcl-6, inducible T-cell costimulator (ICOS) and CD28[107]. As for GC B cells, both the receptors CXCR5 and $S1P_2$ are important for T_{FH} cells to localize in the GC[108-110]. The surface markers $CD4^+CD44^+CD62L^-CXCR5^+PD1^+$ define T_{FH} cells. Activated T cells such as the T_{FH} cells express CD40L that interacts with CD40 on the surface of GC B cells. A block in this interaction abrogates the GC reaction[111]. CD40-CD40L interaction is also important for isotype switching of B cells[112]. CD40 supports the early GC formation while expansion of dark zone B cells occurs in the absence of CD40 signaling in B cells[113]. Two important interleukins for the GC response produced by T_{FH} are IL-21 and IL-4 (Fig. 3). IL-21 is important for T_{FH} differentiation and GC B cells. IL-21 influences the GC B cells to express Bcl-6 and it also promote B lymphocyte-induced maturation protein (Blimp-1) expression and plasma cell formation. These very separated

functions make IL-21 important in both follicular and extrafollicular responses[114]. The contact between a T_{FH} cell and a GC B cell is short-lived, even if the MHC class II molecules present a high level of cognate peptide. The cell-cell contact increases a transient Ca^{2+} flux in the T_{FH} cell that is associated with expression of IL-4 and IL-21, which leads to an advantage for high-affinity B cells[107,115,116]. When $ICOS^{+}$ T_{FH} cells interact with B cells expressing ICOSL, the T_{FH} cells produce IL-4. ICOSL-deficient mice have a normal GC formation but impaired selection of high-affinity B cells[117]. SLAM-associated protein (SAP) expression by T_{FH} cells is important for stable T-B cell interaction. SAP interacts with the intracellular tail of membrane bound signaling lymphocytic activation molecule (SLAM) family members important for several different immunomodulatory functions such as adhesion and costimulation[118]. SAP-deficiency leads to reduced GC, plasma cell and memory B cell formation while extrafollicular responses are intact[119]. GC B cells with high amount of peptide-MHC class II presented to the cognate T_{FH} cells have also more cell divisions and higher affinity maturation compared to GC B cells with less antigen on the surface[120]. In the initial T and B cell interaction at the T-B cell boarder, the T cell follows one B cell as a conjugate. These T-B cell conjugates can last up to 60 minutes while interaction between T_{FH} cells and B cells in GCs are short and transient[116,121]. The T_{FH} cells continue to another target GC B cell after the transient interaction[116]. GC B cells are clonally expanding within one GC, while T_{FH} cells are able to leave a GC and emigrate to a neighboring GC. In the next GC the newly migrated T_{FH} cells are able to participate in selection of GC B cells with high peptide-MHC class II titers[122]. There are two limiting factors, in combination, for GC B cell expansion, first the antigen-specificity of the BCR and second the help from the T_{FH} cells. B cells have to recognize and compete with other B cells for the soluble antigen or antigen presented on FDCs and get stimulation from T_{FH} cells. If the T_{FH} cells are able to invade ongoing GC reactions and interact with preexisting GC B cells for selection the specificity of the TCR may be polyreactive and not selecting only one GC B cell clone for expansion.

1.2.5 B cells

GC B cells can be identified by expression of the death receptor CD95 (Fas), n-glycolylneuraminic acid (the ligand of antibody GL-7) and binding to peanut agglutinin (PNA). These cells are one of the fastest proliferating cell types known, and have an estimated cell-cycle time between 6-12 hours[88,89]. When B cells in the follicle gets activated by antigens they upregulate CCR7 for migration towards the ligands CCL19 and CCL21 expressed in the T cell zone[121]. CCR7-deficient B cells gets to the B cell follicle but have a reduced capacity to migrate to the T cell zone[70]. For B cells to act normal and localize in the GC, regulation of two chemotactic G protein–coupled receptors, $S1P_2$ and Epstein-Barr virus induced gene 2 (Ebi2) is important. $S1P_2$ is important for GC homeostasis. GC B cells have a higher expression of $S1P_2$ than follicular B cells. Deletion of $S1P_2$ leads to uncontrolled growth of GCs and diffuse large B cell lymphoma with GC origin[123,124]. GC B cells downregulate Ebi2 after activation and migrate towards the center of the follicle where the

FDCs are located. Ebi2-deficient B cells localize in the middle of the follicle without activation. In Ebi2-deficient mice migration of plasmablasts towards the extrafollicular foci are lost and the early antibody production is reduced[125-127]. Upregulation of Ebi2 make the activated B cells move to the outer part of the follicle and to extrafollicular areas where the Ebi2 ligand, 7α,25-dihydroxycholesterol is increased[128].

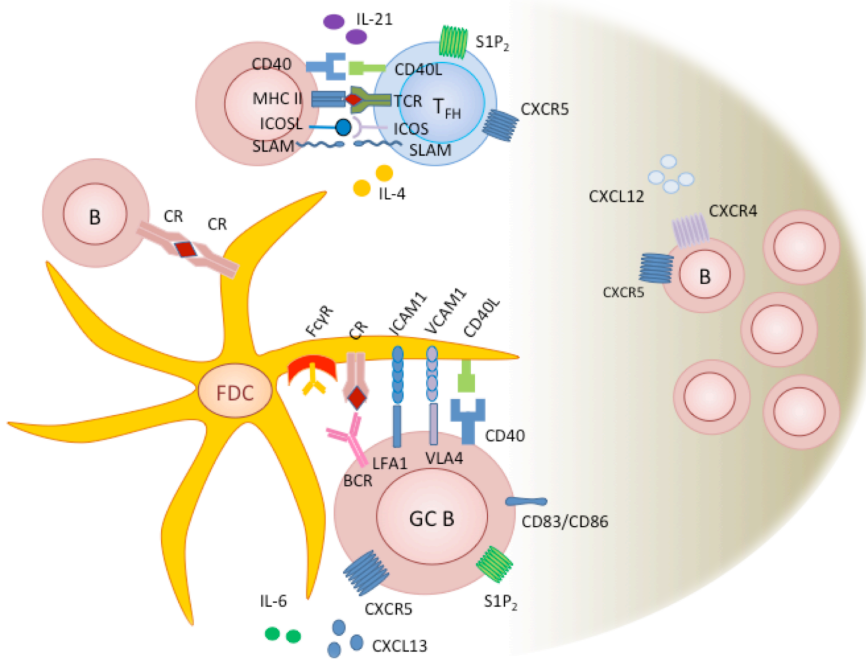

Figure 3: Schematic picture of the germinal center. Important receptors and ligands discussed in the text are highlighted. *Illustration by Emelie Risberg.*

Even if the light zone and the dark zone themselves are markedly different it is difficult to distinguish the B cells from the zones from each other by only looking at their morphology. In the splenic GCs the light zone is strategically placed towards the marginal sinus where the antigens enter whereas the dark zone is closer to the T cell zone (Fig. 2). The classical model of a GC reaction is that the B cells, centroblasts, proliferate vigorously and move from the dark zone to the light zone, become centrocytes which are less active and get selected for their antigen recognition[129]. New research has strengthen this theory but also added that the B cells can migrate in between the zones, with a net movement favoring from dark zone to light zone migration. During a 6 hours time lapse 50% of the dark zone B cells migrate to the light zone, while only 10% returns to the dark zone[130,131]. For the migration to occur chemokines and their receptors are needed. The FDCs covers a big area of the light zone, producing

CXCL13, which attracts B cells expressing the CXCR5 receptor. Both the chemokine and the receptor are necessary for normal accumulation in the light zone. For B cells to migrate to the dark zone CXCR4 expression is required and its ligand, CXCL12, potentially produced by local stromal cells in the dark zone. B cells in the dark zone have increased CXCR4 expression compared to B cells in the light zone. In contrast there is no difference of CXCR5 expression on B cells in the different GC compartments[132,133]. To be able to differentiate light zone B cells from dark zone B cells in mice Victora and colleagues identified the activation markers CD83, CD86 and CXCR4. CD83 or CD86 in combination with CXCR4 can separate light zone and dark zone B cells from each other, respectively[130] (Fig. 3). In 2012 the same research group defined that the same markers can be used to detect GC B cell polarization in human tonsils [134]. CXCR5-deficient mice have a severely impaired splenic architecture with lack of B cell follicles; instead the B cells accumulate close to the marginal sinus. After immunization with TD antigen GC-like structures are formed in close relation to the FDCs in the T cell zone in CXCR5-deficient mice. Even if the structures have no light zone/dark zone polarization the B cells go thorough high-affinity maturation. This shows that SHM can occur independently of GC polarization[135]. SHM in B cells can occur extrafollicular without CD40 signaling for example after immunization with TI antigen, but the SHM rate is not as high as after TD antigen immunization[136]. In contrast to CXCR5-deficiency, B cells lacking the guanine exchange factor (GEF) dedicator of cytokinesis 8 (DOCK8) have formation of GCs at the correct location, but fail to produce B cells with high affinity for the antigen. DOCK8-deficient B cells have impaired recruitment of ICAM1 to the BCR-associated immunological synapse. Even if no other BCR signaling related aspects are found altered, the DOCK8-deficient B cells are unable to sustain GCs, similar to BAFF/BAFFR-deficient mice, and get high-affinity mutations[137].

1.2.5.1 Bcl-6 in B cells

The transcription factor B-cell lymphoma 6 (Bcl-6) works as a transcriptional repressor. Mice lacking Bcl-6 are not able to form GCs or high-affinity plasma cells. Bcl-6 works in several complex ways to maintain GC phenotype of the B cells. Bcl-6 represses Ebi2 expression to localize the activated B cell in the follicle[125]. GC B cells are highly susceptible DNA damage by AID, which is required for SHM. To let the GC B cells tolerate this, Bcl-6 represses factors such as the cell-cycle controlling p53[89,138]. Bcl-6 keeps the GC B cell in a proapoptotic state by repressing the antiapoptotic protein Bcl-2[139]. Bcl-2 transgenic mice that have a block in cell death have increased titers of high-affinity memory B cells, while leaving the plasma cell number unchanged[140]. Blimp-1 is expressed mainly in plasma cells[141], and its expression is regulated by Bcl-6 leading to no plasma cell formation as long as Bcl-6 is activated[142,143].

1.2.5.2 AID, CSR and SHM

In 1958 clonal selection was revealed by studying single cells and their incapacity to produce antibodies with specificities to more than one epitope[144]. This clonal selection occurs in the GC reaction via **somatic hypermutations** (SMHs) in the V(D)J region of the BCR. During B cell development, RAG-dependent V(D)J recombination expands the possible binding specificities for antigens of the BCR (Fig. 4). To be able to increase the affinity for specific antigens additionally the BCR goes through SHM. The area on the BCR with antigen recognition, the antigen-binding fragment (Fab), includes three regions with most antigen contact, the complementarity-determining regions (CDRs). These regions are also the once with highest frequency of mutations[145](Fig. 4).

The class of the BCR/antibody is defined by the IgH chain's constant region (Fc). The different isotypes an immunoglobulin can be is IgD, IgM, IgG, IgA and IgE. Between the constant regions for the different heavy chains are switch (S) regions located. The **class switch recombination** (CSR) occurs between these S regions and the DNA between these regions is cut out as an extrachromosomal DNA switch circle. **Activation-induced cytidine deaminase (AID),** deaminates cytosines in both the donor and acceptor S regions, which induces a double break in the DNA (Fig. 4).

During CSR the Fab regions of the immunoglobulin remains the same. Depending of what kind of stimuli the B cell receives different isoforms are produced. CD40 and TLR4 signaling are crucial for murine B cells to undergo CSR. LPS, the ligand for TLR4, skews the switching to a TI antigen response and production of IgG2b and IgG3. Stimulation with both IL-4 and CD40 or LPS induce IgG1 and IgE, and CD40 or LPS with interferon-γ (IFNγ) and transforming growth factor-β (TGFβ) make the B cells switch to IgG2a and IgA respectively[112,146,147]. CD40- or CD40L-deficiency leads to hyper IgM (HIGM) syndrome with marked reduction of switched isoforms[148].

The key enzyme for both CSR and SHM is AID. AID deaminates cytidine residues, both in V(D)J and switch regions of the immunoglobulin gene. The deamination of DNA specific for GC B cells changes deoxycytidine (C) into deoxyuridine (U) and transforms C:G pairs into U:G mismatch[149]. The deoxyuridines are removed by uracil DNA glycosylase (UNG) leading to single-strand breaks (SSBs). Since it has to be a double-strand break (DSB) for CSR to occur, it could either be two SSBs with close proximity on opposite DNA strands that forms the DSB naturally or during the mismatch repair of DNA the SSB is converted to DSB[150]. Interestingly, Bcl-6-deficient mice have a great reduction of AID and UNG expression. It might even be that Bcl-6 directly regulates UNG by being able to bind to the *UNG* locus[143]. Bcl-6-deficient mice do not form GCs and AID-deficient B cells have large spontaneous GC formation in spleen, lymph nodes and payer's patches. After stimulation with TI antigen LPS *in vitro* AID-deficient B cells are unable to undergo CSR. CSR and SHM is also lacking after TD antigen immunization in AID-deficient mice[151].

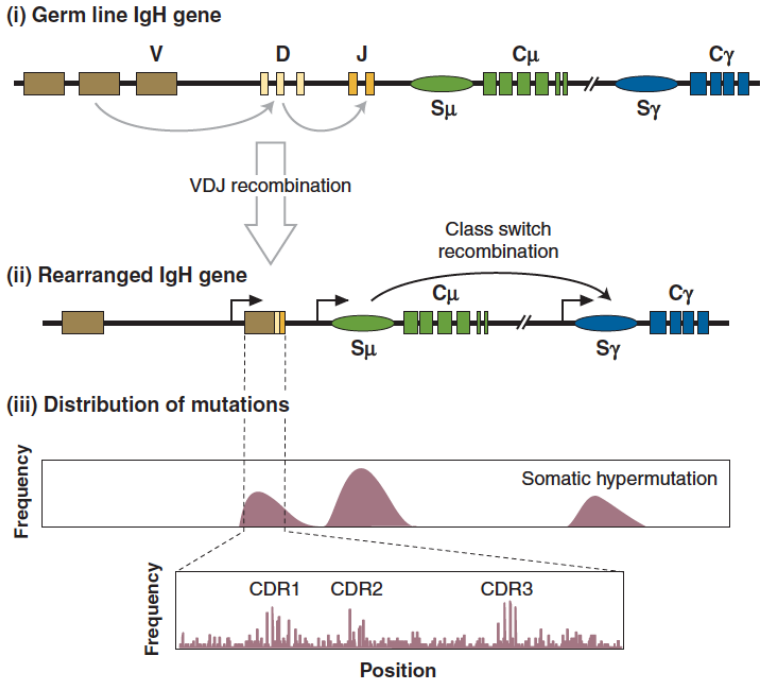

Figure 4. Antibody gene diversification. Schematic representation of the diversification processes of the IgH chain of the BCR locus. (i) The RAG-dependent V(D)J recombination occur in the bone marrow in immature B cells and is followed up in the spleen in GC B cells with (ii) the AID dependent class switch recombination and somatic hypermutation. (iii) The peaks represent highly mutated areas that differ from the germline sequence. The complementarity-determining regions (CDRs) are the areas with the highest amount of SHM in the *IgH* gene[145] (from Di Noia, J.M. & Neuberger, M.S. *Annual review of biochemistry* **76**, 1-22 (2007)).

Although AID has its function in the nucleus, the biggest proportion of the AID protein is found in the cytoplasm. Since AID induces mutation in a million-fold higher rate than normal mutations, nuclear localization can lead to mutations in oncogenes, even if the AID off-target mutation rate is restricted[152,153]. The C-terminal portion of the protein is absolutely required for CSR, and mutations in this region lead to absence of CSR but SHM can still occur[154].

There are several mutations in the *AID* gene leading to HIGM. There is one mutation that is frequently detected in HIGM patients in amino acid position 112, leading to an arginine substitution to either histidine or cysteine[145]. This mutation in position 112, is the corresponding mutation in humans that we found in mice presented in **paper IV**.

1.2.5.3 Memory B cells and plasma cells

B cells that have interacted with cognate T cells can either decide to stay in the follicle and participate in the GC reaction, or they leave towards the marginal zone and become short-

lived plasma cells in the extrafollicular foci. These plasma cells express the transcription factors Blimp-1 and X-box binding protein 1 (XBP-1), but lack SHM and high affinity for the antigen. Compared to B cells in GC reaction, Ebi2 is important for B cells that are involved in the extrafollicular response[155]. Except for extrafollicular responses, memory B cells and long-lived plasma cells come from the GC reaction. One important difference between the two cell types it that memory B cells keep their BCR on the cell surface while plasma cells downregulate their BCR to become high producers of secreted antibodies. Compared to many other mature B cells, the memory B cells do not require BAFF or cognate BCR stimulation for their survival. Memory B cells can switch away from their initial recognition for the antigen, which makes them resting and not dependent on antigen recognition [156].

In 1948, Astrid Fagraeus proposed that antibodies are produced by plasma cells and the B cell stages before becoming plasma cells were called immature plasma cells[157]. Now we know that the plasma cell fate is determined in the GC B cells. Plasma cells downregulate CCR7 and CXCR5 and upregulates CXCR4 to be able to leave the follicle. Stromal cells in the bone marrow express CXCL12, which makes the $CXCR4^+$ plasma cells home to the marrow[158]. Plasma cells in the bone marrow have gone through SHM and have high-affinity maturation, compared to memory B cells in the spleen with lower affinity for the antigen[159].

The master regulator of plasma cell differentiation is the transcription factor Blimp-1. Cytokines such as IL-2 and IL-5 are important inducers of Blimp-1 upregulation[141]. Blimp-1 represses genes that are important for cell proliferation, such as the proto-oncogene c-Myc and Bcl-6, leading to cell-cycle arrest and terminal differentiation of the B cells[160-162]. The transcription factor Pax5 that is important for B cell commitment in the bone marrow might also have an important role in plasma cell formation. Pax5-deficient mature B cells have downregulated Bcl-6 and upregulated Blimp-1 and XBP-1 expression, which could indicate that Pax5 inhibits plasma cell formation[163]. Pax5 activates Bach2, which supports proliferation and CSR by repressing Blimp-1. Another challenging multifunctional transcriptional regulator upregulated by Pax5 is interferon regulatory factor (IRF) 4. IRF4 is required for GC formation, CSR and also for plasma cell formation, most probably in a dose dependent manner[164]. Systemic lupus erythematosus (SLE) patients have elevated plasma cell titers and also increased Blimp-1 expression[165]. Lupus prone mice, MRL-Fas(lpr), one mouse model for SLE, have increased Blimp-1 expression in peripheral blood and silencing of Blimp-1 lead to reduced anti-DNA antibodies[166].

Even if plasma cells have the intracellular machinery for antigen presentation and they express MHC class II and costimulatory molecules they are not able to induce activity of T_{FH} cells. This regulation of response is thought to be a negative feedback loop to control ongoing B cell immunity to both foreign and autoantigens[167]. Preexisting antibodies compete with new GC B cells for binding to the antigen on FDCs and thereby increasing the selectivity for new high-affinity B cells [168]. Next, I will present how aberration in the cytoskeletal proteins such as WASp, N-WASp and Cdc42 in B cells may have impact on GC responses and the immune system.

1.3 WISKOTT-ALDRICH SYNDROME

Wiskott-Aldrich syndrome (WAS) is a rare potentially life-threatening X-linked primary immunodeficiency. WAS is characterized by a clinical triad of thrombocytopenia, eczema and immunodeficiency. The frequency of WAS is 1-10 per million males. Without treatment the outcome is poor, with a median survival of 15 years[169]. Since the first bone marrow transplantation made more than 40 years ago the outcome has become better and better. The only way to cure WAS is by hematopoietic stem cell transplantation or gene therapy, currently in clinical trial[170]. Up to 70% of WAS patients develop autoimmunity[171,172].

The protein causing WAS was identified 1994, and was named Wiskott-Aldrich syndrome protein (WASp)[173]. Cells from WAS patients and WASp-deficient mice show reduced cytolytic, phagocytic and antigen presenting functions, together with reduced antibody responses upon foreign antigen challenge[174]. WAS has been consider a T cell disorder, with only primary defects in T cells leading to secondary effects on other cell types[175]. In this part of the introduction I have focused on the role of WASp in B cells, since we know today that all hematopoietic cells have intrinsic dysfunctions in the absence of WASp.

1.3.1 WASp family members in B cells

The **Wiskott-Aldrich syndrome protein (WASp)** is an actin polymerizing protein belonging to WASp protein family. The WASp family of proteins includes eight proteins where neural WASp (N-WASp) is the most related protein to WASp. N-WASp is homologous to WASp, with 72% homology on the amino acid level, leading to 80% identity of the functional domains[176]. WASp expression is restricted to hematopoietic cells, while N-WASp is ubiquitously expressed, leading to that N-WASp-deficiency is embryonic lethal[177]. WASp is a 502 amino acid protein and similar to N-WASp have five functional domains: the WASp homology 1 (WH1) domain, a basic region, a GTPase-binding domain (GBD), a polyproline-rich region and the C-terminal VCA domain (Fig. 5). The WH1 domain binds the WASp-interacting protein (WIP), which stabilizes the WASp protein and the GDB binds the RhoGTPase Cdc42 which activates WASp and N-WASp[178,179]. The conserved C-terminal region, verprolin homology (V, or WH2 motif), cofilin homology (C, or central) and acidic (A), build up the **VCA domain.** The V domain binds the actin monomers, while the A domain binds the actin-related protein 2 and 3 (Arp2/3) complex essential for the actin polymerization and the C domain has binding capacity to both the actin monomers and the Arp2/3 complex (reviewed in [178,180]) (Fig. 5).

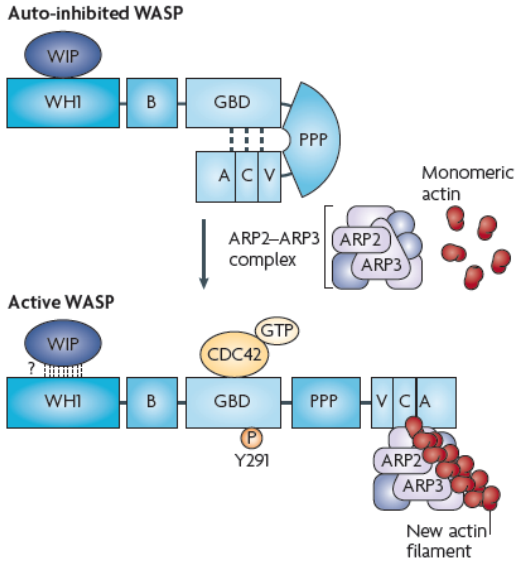

Figure 5. Wiskott-Aldrich syndrome protein (WASp) gets activated and opens up from the auto-inhibited conformation when the GTPase Cdc42 binds to the GTPase binding domain (GBD). The Arp2/3 complex binds to the VCA domain and monomeric actin becomes polymerized actin[178] (from Thrasher, A.J. & Burns, S.O. *Nature reviews. Immunology* **10**, 182-192 (2010)).

1.3.2 WASp and N-WASp

WAS patients have normal IgG titer in blood, while there is a decrease of IgM and increase of IgA and IgE titers[181]. WASp patients and WASp-deficient mice have similar defects in populating the splenic marginal zone[174,182-184]. As described above, there are some crucial steps for MZ B cell development. Btk activation upon antigen binding to the BCR is required for phosphorylation of WASp. Btk activates a GEF for the cell division control protein 42 (Cdc42), which activates WASp that leads to actin polymerization and spreading. In contrast, by letting SHIP-1 suppress Btk activation, WASp activation is inhibited promoting contraction of the B cell. SHIP-1-deficient B cells have increased accumulation of polymerized actin and WASp phosphorylation in a Btk-dependent manner, leading to cell spreading[185]. Interestingly, N-WASp phosphorylation is increased, rather than decreased as for WASp, in Btk-deficient B cells. The opposite relation occurs in SHIP-1-deficeint B cells, where N-WASp is decreased while WASp is increased. This suggests that WASp and N-WASP activation have different activation pathways and may counteract each other during specific conditions[186].

Until today, only two papers are published on Cdc42 in B cells[187,188]. The data from these two publications will be analyzed and compared to our findings in **paper III** in the section ”Present study”. Similar to DOCK8-deficient mice, WASp-deficient mice have reduced ability to form immune synapses upon BCR signaling and ICAM1 stimulation[183]. MZ B cells

upregulate LFA-1 and interaction with ICAM1 mediates B cell adhesion, which is important for MZ B cell localization [189]. The other important factor for MZ B cell development is Notch signaling. WKO B cells have reduced expression of one of the target genes (Hes1) in Notch-signaling. In contrast, in bone marrow chimeric mice where only B cells lack WASp there is no difference in Hes1 expression, indicating that the reduced MZ B cell numbers are not because of defect Notch-signaling[183].

Mature lymphoid cells expressing WASp have a selective advantage over WASp-deficient cells in both human and mice[182,183]. This positive selection of WASp expression cells is not found in the myeloid compartment[190]. The locomotion capacity is reduced in WASp-deficient DCs, macrophages, T cells and B cells[191-194]. T cells deficient in both WASp and N-WASp have a more severe phenotype than WASp-deficient T cells. The double-deficient T cells show marked block in development resulting in reduced T cell number in the periphery, and the surviving cells have migratory defects towards CXCL12 and CCL19[195]. This finding in T cells was one reason that made us interested in investigating the role of WASp and N-WASp in B cells, resulting in **paper I** and **II.** If there are defects in functional migration capacity of cells, the interaction and stimuli between cells will lead to secondary defects in the target cells. Westerberg et al. showed 2008 that WASp-deficient MZ B cells have reduced migration capacity towards S1P as well as reduced $S1P_1$ expression in MZP-T2 population and reduced $S1P_3$ expression in the MZ B cell population, resulting in reduced homing capacity to the marginal zone[182].

1.3.3 The redundant function of actin regulatory proteins

WASp and N-WASp reside in an auto-inhibited conformation in resting cells, and the conformation opens up upon extracellular stimuli, such as via the BCR. When Cdc42, binds to the GBD on WASp and N-WASp, the VCA domain is released from the GBD and the basic domain, located next to the GBD (Fig. 5). **Cdc42** is a guanine nucleotide-binding protein that gets activated by binding of GTP. The switch from GDP to GTP is facilitated by GEFs, such as DOCK8. Cdc42 is within the small **RhoGTPase family**, together with 22 other RhoGTPases[196]. Ras-related C3 botulinum toxin substrate 1 (Rac1) and Rac2 are two other small RhoGTPases important for B cell development. Rac2-deficiency leads to a loss of B1 B cells and MZ B cells, while Rac1-deficient mice have no altered B cell phenotype. Mice with both Rac1 and Rac2 deleted have a more severe phenotype with a B cell developmental block already in transitional B cells since both Rac1 and Rac2 are important for transduction of BAFFR signals[197]. Rac can be activated, as Cdc42, by DOCK8. Both Rac and Cdc42 can be activated by other GEFs as well, such as VAV1-3. Mice deficient in VAV1 has slightly reduced B1 B cell numbers, while other B cells develop normally. VAV1 and VAV2-deficient mice have reduced numbers of both follicular and MZ B cells, and mice lacking all three, VAV1-3, have an even worse phenotype (described in a review by Tybulewicz et al.)[196]. The effector protein of Rac is WAVE, which also belongs to the WASp family of

proteins. WAVE and WASp/N-WASp induce different kind of protrusions of the plasma membrane, lamellipodia and filopodia structures, respectively. Lamellipodium is larger protrusions filled with polymerized actin, while filopodium are thinner and protrudes from the cell body or from the lamellipodium[198]. Activation of Cdc42 and Rac1 induce filopodia and lamellipodia, respectively, in B cells[194]. Even if the different RhoGTPases and effector proteins give rise to different actin containing structures, it is the MZ B cells that are first and most affected when actin-regulating proteins are deleted. The redundancy found in Rac1 and Rac2 and in VAV-family members is relevant to the redundant function of WASp and N-WASp we see in **paper II**, and might also influence the results in **paper I** and **paper III**.

1.3.4 WASp and autoimmunity

By studying autoimmunity in WASp-deficient mice the knowledge of autoimmunity in primary immunodeficiency has increased. Up to 70% of WAS patients suffer from autoimmune disorders such as antibody-mediated cytopenia, arthritis, skin vasculitis, glomerulonephritis and inflammatory bowel disease[172,178]. Older WASp-deficient mice develop autoimmune features such as colitis, anti-nuclear, anti-dsDNA and anti-platelet antibodies[181,190,199-201]. Since all WASp deficient lymphocytes are defect, both T and B cells have a role in autoimmunity in WAS. T regulatory (T_{reg}) cells are important for immune suppression, and disruption of functional T_{reg} cells is a primary cause of autoimmune diseases[202]. Both WAS patients and WASp deficient mice have reduced T_{reg} cell suppressive function which could explain the risk of development of autoimmunity[190]. T_{reg} cells expressing WASp have a selective advantage, resulting in normal T_{reg} cell population in female WASp heterozygous mice. Interestingly, these mice have increased autoreactive antibodies compared to wild type mice even if T_{reg} cells are normal, suggesting a cell-intrinsic dysfunction in WASp-deficient B cells[199]. Two recent publications have investigated the role of WASp in B cells in breaking self-tolerance and autoimmunity in a setting of normal T-cell function. One study uses bone marrow chimerice mice to have expression of WASp in all cells except B cells, while the other study uses gene targeted conditional knock out mice[199,203]. The following section is a summary of the two studies.

Mice with conditional WASp-deletion in B cells are in many aspects similar to complete WASp-deficient mice, presenting an intrinsic B cell dysfunction in WASp-deficient mice. WASp-deficient follicular and GC B cells are hyperproliferative and hyperresponsive to anti-IgM and TLR stimulation. When hyperresponsive WASp-deficient B cells get the sufficient help from wild type cells, e.g. DCs and T cells, the phenotype is more severe than if all cells lack WASp. The B cell conditional WASp-deficient mice have reduced marginal zone, reduced humoral response to TI antigens, increased IgM titers in serum and autoantibody production similar to the complete WASp-deficient mice. In addition, the B cell conditional WASp-deficient mice have also spontaneous GC formation and renal histopathology associated with autoantibodies[199,203].

2 THE PRESENT STUDY

2.1 AIM

The general aim of the present study was to elucidate the role of primary immunodeficiency-associated proteins in humoral response to both foreign and autoantigens.

The specific aim for each individual paper was:

Paper I – To clarify the specific role of actin-regulating proteins WASp and N-WASp in B cell activation to foreign antigens and autoantigens.

Paper II – To elucidate the unique and redundant activity of WASp and N-WASp for B cell development and function.

Paper III – To identify the specific role of the RhoGTPase Cdc42 for B cell activation and the humoral response.

Paper IV – To characterize the recessive and spontaneous AID mutation causing Hyper IgM syndrome in mice.

2.2 MATERIAL AND METHODS

This section explains two important methods used in this thesis. A more detailed description of all methods used can be found in the individual papers.

2.2.1 Mice and the Cre/loxP system

In **papers I-III**, the genes *N-WASp* and *Cdc42* were deleted conditionally from B cells by using the Cre/loxP system. The advantage of this system is the ability to inactivate genes in specific cells and/or at a specific time, depending on under what promoter the Cre recombinase is located. By mating Cre-expressing transgenic mice with mice having the gene of interest flanked with recombinase recognition sites, loxP sites "floxed", the Cre recombinase can cut out the region between the loxP sites[204]. By using Cre-ERT2 mice, as in **Paper III**, the gene inactivation can occur during controlled time points by giving the mice tamoxifen by gavage. Tamoxifen is an antagonist for the estrogen receptor ERT2 normally located in the cytoplasm. Binding of tamoxifen to Cre-ERT2 makes the complex relocate to the nucleus for recombination. Important to think about when using Cre deletion of genes is that the gene is normally expressed until the promoter where Cre is located under gets activated. Since the protein of interest is expressed at normal levels before the Cre recombinase is activated it is important to take the half-life of the protein into consideration, and measure the deletion of the protein in different maturation stages of the cells. When Cre is located under the CD19 promoter it is know that the Cre recombinase deletes the floxed target gene in 75–80% in bone marrow-derived pre-B cells and 90–95% in splenic B cells[205]. In **paper I** and **II** we have used heterozygote $CD19^{+/Cre}$ which contains one functional CD19 allele and is phenotypically normal. The following mice were heterozygous for Cre – **paper I** and **II**; N-$WASp^{fl/fl}CD19^{+/Cre}$, **paper III**; $Cdc42^{fl/fl}CD23^{+/Cre}$ and $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$.

2.2.2 NP-KLH as antigen

The advantage of using NP-KLH as antigen is the well-characterized clonal expansion of B cells after immunization, without usage of transgenic mice. NP-KLH gives rise to high-affinity antibodies with specificity for NP. More than 50% of the NP-specific B cells express the IgH V_H1-186.2 paired normally with a λ light chain[206,207]. The specific mutation in V_H1-186.2 induced in high-affinity BCRs/antibodies are in amino acid position 33, a tryptophan to a leucine exchange (W33L) and the antibody with this specific mutation was from the beginning named 3B44. Antibodies can have high affinity to NP, for example 3B62, but in those cases no specific high affinity mutation have been found[208]. Amino acid 33 is within the CDR1. We have determined the high-affinity mutation rate in wild type, WKO and cDKO mice in **paper I**, and in AID^{R112H} mice in **paper IV**, by sequencing the CDRs. When looking for different mutations, alterations from the germline sequence are mostly found in the CDR3

region. This region covers the junctions between the V, (D), and J segments thus becoming the most variable part. In short the procedure is as follow; a polymerase chain reaction (PCR) using a forward primer binding to the specific V_H1-186.2 and a reverse primer binding to the constant region of interest, for us Cμ and Cγ1 in **paper I** and **paper IV**. The PCR products were cloned into TOPO TA for amplification and the products were sequenced and compared to the results of the corresponding germline sequence.

2.3 RESULTS AND DISCUSSION

2.3.1 The discovery of a new spontaneous AID mutation in mouse

For the ease of discussion I will first discuss **paper IV** followed by **papers I-III**.

In a separate project Lena Ström and Torkild Visnes, co-authors on **paper IV**, were investigating mice heterozygous for a gene-trap mutation in *nipbl* (Nipped-B like) NIPBL, is a protein complex important for loading the cohesin complex to the chromosomes[209]. We screened these mice for antibody titers and performed Ig-class switching experiments *in vitro*. We found that some of the control mice were incapable of producing class switched antibodies both *in vivo* and *in vitro*. After some investigations, this finding led to **paper IV**. The control mice, *nipbl*$^{+/+}$, were littermate controls to *nipbl* $^{+/-}$ mice, which showed normal titers of switched antibodies.

As described above, Minghui He examined the class switching capabilities *in vitro* using splenic B cells from *nipbl*$^{+/-}$ mice and as littermate control she used B cells from *nipbl*$^{+/+}$ mice. I investigated total antibody titers in serum from the remaining mice in the same litter by enzyme-linked immunosorbent assay (ELISA). In one of the control mice our collaborators could not detect any CSR, while I found three additional mice without any detectable IgG but elevated titers of IgM antibodies in serum. These two experiments were the start of an interesting and fun parallel project during Minghui He's and my graduate studies.

We bred mice with no IgG antibody titers with each other hoping to obtain more mice with a similar phenotype. The offspring had no detectable IgG and relatively higher IgM antibody titers than wild type mice. Since the mice originated from an outbred strain (CD1), we backcrossed the mice to a C57Bl/6 background for further analysis. After one generation of backcrossing to C57Bl/6 background, the hyper-IgM (HIGM) phenotype was clearly detectable, and we could show that the phenotype was hereditary. The mice were screened by ELISA and categorized by the presence or absence if HIGM phenotype. We first thought of investigating the mRNA expression from genes that can cause HIGM syndrome, e.g. genes encoding for CD40, CD40L, AID or UNG[210]. Since we observed a defective antibody production by B cells in *in vitro*, we hypothesized that the defect was B cell intrinsic. We therefore excluded CD40L-deficiency. CD40 is needed to sustain GC reaction[139] and since we detected spontaneous GC formation we also excluded CD40 from the list of potential missing proteins. The levels of *aicda* and *ung* mRNA in HIGM B cells were similar to that of wild type B cells. Next we sequenced cDNA coding for the different proteins, with *aicda* as a first candidate. Strikingly, we found a mutation in position 427, leading to a replacement of arginine to histidine in amino acid position 112 of the AID protein. The HIGM mouse was therefore given the name AID^{R112H}. This mutation is located in the linker region between the cytidine-deaminase motif and the apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 1 (APOBEC1) – domain. This mutation has been found in several unrelated

HIGM patients but until now there has been no animal model replicating this genetic mutation of HIGM[147,211]. Next we immunized AID^{R112H} mice with NP-KLH, and found that no high-affinity B cells were formed, but some mutations in other positions could still be detected (data not shown).

The behavior of the mutated protein we detected was similar to the wild type protein. AID^{R112H} protein was located in the nucleus and was able to bind to the switch regions required for CSR. This suggested that the AID^{R112H} mutation, which is located in between two important regions in the *aicda* gene, makes the protein catalytically inactive. One very interesting phenotype found in AID-deficient mice, and in our AID^{R112H} mice, is the spontaneous formation of GCs. In AID-deficient mice the sustained GC formation could be explained by reduced apoptosis detected in B cells lacking AID[212]. Interesting future projects include more molecular studies on AID^{R112H} B cells. It would be of outstanding interest to measure the amount of Bcl-6, Bcl-2, IRF4, Blimp-1 and other factors known to play a role in GC and plasma cell formations. How these factors regulate AID expression has been studied, but it is to my knowledge unknown if and how AID regulates the factors. Experiments with bone marrow chimeric mice with B cells lacking or expressing AID show that AID-deficient B cells have an advantage in occupying the GC[212]. By making chimeric mice with bone marrow from both AID-deficient mice and AID^{R112H} mice we could investigate if our AID^{R112H} protein would make the B cells behave more like a wild type B cells or as AID-deficient B cells.

Finally, I would like to speculate – how come a spontaneous mutation in AID is allowed to be inherited by mice with a mutation in *nipbl*? In all mice screened, the HIGM phenotype was found only in *nipbl*$^{+/+}$ mice. Fewer mice with the genotype *nipbl*$^{+/-}$ were born compared to mice with the genotype *nipbl*$^{+/+}$ and the AID^{R112H} mutation may be derived from the *nipbl*$^{+/-}$ mouse. If the AID^{R112H} mutation is derived from the *nipbl*$^{+/-}$ mouse it may be that it was just by chance that no *nipbl*$^{+/-}$ mouse had detectable HIGM phenotype. From where the AID^{R112H} mutation originates could be investigated more thoroughly. NIPBL is important for correct loading and function of cohesion, which is important for chromatid and gene regulation but also for DNA repair[213]. Could it be beneficial to lack functional AID if the *nipbl* protein is not functional? Yes! Likely, since NIPBL is important for DNA repair and AID is causing double strand breaks in DNA it is possible that *nipbl* $^{+/-}$ mice that also lack functional AID have a survival advantage. Deficiency in functional NIPBL causes the developmental disorder Cornelia de Lange syndrome (CdLS). Recurrent infections are a significant cause of mortality in CdLS, and 30% of the patients in one study had antibody-deficiency syndrome[214]. Could it be that the CdLS patients with antibody-deficiency syndrome actually have mutation in their *AICDA* gene? If AID mutation and/or dysfunction are partly the cause of the recurrent infections leading to mortality of the CdLS patients I think it would be of great interest to investigate AID expression and function, as well as potential mutations, in the patient's B cells. One study from 2013 shows that B cells derived from CdLS patients have reduced sensitivity to DNA damage and reduced CSR[215], which implies that AID expression might be altered in these B cells.

2.3.2 The role of Cdc42, WASp and/or N-WASp in murine B cells

Wiskott-Aldrich syndrome (WAS) is considered a cell-trafficking disease in which all hematopoietic cells show decreased migratory, adhesive and receptor-mediated responses. Since Cdc42 is a RhoGTPase for WASp and N-WASp, we could speculate that similar phenotype should be detected in mice lacking Cdc42 or both WASp and N-WASp, together with a milder phenotype in WASp-deficient mice. In the following results and discussion part I have compared the findings in these mice. We hypothesize that Cdc42, WASp and/or N-WASp-deficiency leads to skewed responses due to intrinsic cell dysfunction but also altered cellular composition of the spleen, contributing to immunodeficiency and autoimmunity. To be able to address this question we have taken the approach to use animal models.

During the course of this work some results from a similar study by Burbage et al. have been published regarding Cdc42-deficient B cells. There are similarities and differences between the two studies, and I will discuss the findings in relation to each other in the following sections.

2.3.3 Nomenclature and mice used in paper I-III

For the sake of simplicity I will begin with the description of the mouse strains used in **papers I-III**. The following abbreviations are used: WKO (WASp deleted in all cells, normal N-WASp), cNWKO (normal WASp, N-WASp deleted conditionally in B cells), cDKO (WKO mice with specific deletion of N-WASp in B cells) and Cdc42KO (Cdc42 deleted conditionally in B cells) (Fig. 6). In **paper III** we have used two different Cre-transgenic mice, $Cdc42^{fl/fl}CD23^{+/Cre}$ and $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ and which one used is stated for each experiment. In **paper I** and **II** a comparison between WKO and cDKO B cells demonstrates the intrinsic role of N-WASp. These mice were of mixed background (129Sv x C57Bl/6), following breeding of WKO mice on a 129Sv background, conditional N-WASP KO mice on a 129Sv background and $CD19^{+/Cre}$ mice on a C57Bl/6 background. Mice with a mixed genetic background (129Sv x C57Bl/6) have increased probability for expression of autoimmune disease[216]. We have not backcrossed the cDKO mice in **paper I** and **II**, to one pure background and this could have an potential effect on the autoimmunity we see in the naive mice, even if the controls are littermates.

Figure 6. Explanation of mice used in **paper I-III.**

2.3.4 Alterations in splenic cell populations

By flow cytometry, we found that the marginal zone precursor (MZP) and the MZ B cell populations were decreased in WKO and cDKO mice. cNWKO mice had normal MZP numbers while a cellular reduction of MZ B cells was detected by flow cytometry. This reduction was not distinguished by immunohistochemistry of spleens from cNWKO mice, where both MZB cells and MZMs appeared similar to those in wild type mice. MZ B cells from WKO mice have increased turnover rate[183], which we also found in both WKO and cDKO mice *in vivo*, most likely compensating for the reduced cell number. There was no difference in cell death, measured *in vitro*, between the strains regardless of stimulation. In comparison to the normal numbers of T1 B cells in WKO and cDKO mice from **paper II**, the $Cdc42^{fl/fl}CD23^{+/Cre}$ mice in **paper III** had reduction in the populations transitional 2 (T2)-MZP and MZ B cells, but an increase of T1 B cells. In contrast, $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ mice had reduced total B cell numbers including all naïve B cell populations, transitional and MZ B cells, in the spleen. This difference between the $Cdc42^{fl/fl}CD23^{+/Cre}$ and $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ mice could be expected since CD23 is not expressed on T1 B cells, and is first expressed in T2 B cells[28]. $Cdc42^{fl/fl}CD19^{+/Cre}$ B cells also differ from WASp and N-WASp-deficient B cells in that they show significantly reduced proliferation and increased apoptosis *in vitro*[187]. In **paper I** we found a decrease in the T2 and T3 populations in WKO and cDKO mice compared to wild type mice. In wild type mice, B cells with autoreactive phenotype become anergic and classified as T3 B cells without further maturation as protection from autoimmunity. Several young lupus prone mouse strains have reduced T3 population, since the autoreactive B cells are not negatively selected for further maturation[50,51]. Since we see increased autoreactivity in our WKO and cDKO mice in **Paper I**, our results support previous data, that T3 B cells have increased self-reactivity.

B cell positioning in the marginal zone is important for clearance of blood-borne pathogens. In experimental models, MZ B cells have been shown to use CD21/CD35 for capturing the TI antigen TNP-Ficoll and shuttle to the FDCs for antigen delivery[71]. In **paper II** we show that WKO mice (as previously published by Westerberg et al. 2008) and cDKO mice have reduced ability to capture and transport TNP-Ficoll, resulting in a reduction of the specific switched antibody response in both strains 7 days after injection compared to wild type mice.

Excess of apoptotic cells is a major contributor to the development of autoantibodies and leads to a potential pool for autoantigens. Cells of the marginal zone are important for taking up apoptotic cells. It is known that WKO bone marrow derived macrophages show reduced and delayed clearance of apoptotic cells[217]. WKO mice have reduced MZMs and cDKO mice have reduction of both MZMs and metallophilic macrophages. However, cNWKO mice have normal macrophage composition in the spleen. The findings of reduced marginal zone cellularity and B cell function in **paper II** raised our interest in investigating the autoimmune milieu in the WASp and N-WASp-deficient mice, leading to **paper I**. We hypothesize that a decreased protective shield of the marginal zone together with intrinsic cell dysfunction leads to altered uptake, response and activation of B cells to autoantigens. To investigate the

localization of dying cells carboxyfluorescein succinimidyl ester (CFSE) labeled syngeneic apoptotic thymocytes were intravenously injected into the mice. As expected the CFSE labeled cells were localized in the marginal zone 30 minutes after injection in wild type mice. Even if WKO and cDKO mice show reduced cellularity in the marginal zone, the injected apoptotic cells were found located in the same area as in wild type mice. Since both macrophages and B cells in the marginal zone were reduced in WKO and cDKO mice, and the CFSE labeled apoptotic cells got captured in the marginal zone, we hypothesized that dendritic cells (DC, $CD11c^+$) may be involved. Indeed, the $CD11c^+$ population was markedly increased in both WKO and cDKO mice, located both in the marginal zone and in the T cell zone. A specific $CD11c^+CD8^+$ DC subset, able to capture and endocytose dying cells, is located in the marginal zone of mice. The subset is $CD8^+$ and $CD11c^+$ while CD205 (DEC205) seems not important for capturing of apoptotic cells[218]. This makes the population specific since a big proportion of $CD8^+$ DCs is $CD205^+$. A potentially good marker for these marginal zone specific DCs could be CD207 (Langerin). $CD207^+CD8^+$ DCs express CD205 depending on location in the spleen, analyzed by immunohistochemistry. $CD207^+$ DCs in the T cell zone express CD205, while the $CD207^+$ DCs in the marginal zone do not[219]. It would be interesting to verify what the increased $CD11c^+$ population we see is in more detail by staining for CD8, CD205 and CD207. The cells could be a mixture of DCs both able and unable to capture apoptotic cells. Alternatively, they could be other cell types such as activated T cells[220].

2.3.5 Spreading, migration and homing capacity

In **paper II** and **III** we measured the ability of IL-4 pretreated B cells *in vitro* to spread and to form long protrusions upon stimuli. Wild type and cNWKO B cells showed a high percentage of spreading while spreading was reduced in WKO, cDKO and Cdc42KO B cells. cDKO B cells had almost abolished capacity to spread and form short filopodia extensions. The filopodia extensions are important for cells to probe their environment, and both WASp and Cdc42-interacting protein 4 (CIP4) are important for their formation. CIP4 is an effector protein of Cdc42 and mediates WASp interaction with microtubules, in turn assembling the cytoskeleton[221]. CIP4 was absent in the protrusions of Cdc42KO B cells while it could be seen in the long protrusions of wild type B cells. Reduction of CIP4 or Cdc42-expression limits microtubule organizing center (MTOC) polarization, promoting immune synapse formation[222,223]. $Cdc42^{fl/fl}CD19^{+/Cre}$ B cells do not have reduced migration capacity towards CXCL12 or CXCL13[187]. In contrast, all WASp-deficient hematopoietic cells have reduced migration capacity, including WASp and N-WASp-deficient T cells [187,191-195]. In **paper II** we show that B cells deficient in WASp and N-WASp have reduced ability to migrate towards CXCL12 *in vitro*.

Since analysis of B cell numbers, architecture of the spleen as well as functional assays of B cells from cNWKO mice showed a phenotype similar to wild type mice, we decided not to

investigate these mice further in **paper II**. However, Dr Wenxia Song was interested in studying the role of N-WASp in BCR signaling and collaboration was set up between our groups. This collaboration led to the recently published finding that N-WASp is a critical negative regulator of BCR signaling[186]. This novel knowledge plays a role for our **paper I** as discussed below.

Since we observed a reduction of spreading and migration towards chemokines in B cells deficient in WASp and N-WASp, we wanted to evaluate the homing capacity of splenic B cells from WKO and cDKO mice *in vivo* in **paper II**. Strikingly, both WKO and cDKO B cells had reduced homing capacity to lymphoid organs compared to wild type B cells. Furthermore, cDKO B cells had a more pronounced reduced capacity to home to the Peyer's patches in the small intestine, both in competition with wild type and WKO B cells. Even if $Cdc42^{fl/fl}CD19^{+/Cre}$ B cells have normal migration capacity *in vitro*[187], $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ B cells had reduced homing ability *in vivo*. By injecting CFSE-labeled B cells intravenously, we showed in **paper III** that less $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ B cells localized to the spleen compared to wild type B cells. The decision to use both immunized donor and recipient mice was based on a previous publication by Westerberg and Severinson on WKO mice, where no homing of WKO B cells from unimmunized mice to the spleen was detected[174]. The result of reduced homing capacity of $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ B cells to the spleen in adoptively transfer experiments can be compared to data from experiment with hen egg lysozyme (HEL)-specific $Cdc42^{fl/fl}Mb1^{+/Cre}$ B cells by Burbage et al. Their HEL-specific B cells lacking Cdc42 had similar migration to the draining lymph node as wild type HEL-specific B cells in HEL immunized mice. There are some technical differences between the homing experiments performed. First, the amount of transferred B cells might be different; we injected approximately 10^7 B cells per mouse while the amount of injected cells is not stated in the article from Burbage et al. If fewer cells are injected, the importance of homing capacity might be clearer. Second, the administration route of the antigen affects the organ to which injected cells home. We used intravenously injection of cells while their cells were intravenous injected but the antigen was administered in the footpad[188]. The location of the popliteal lymph node makes it possible for intravital imaging of live cell interactions in situ. I believe it would be of great interest to know whether the CCR7-dependent migration *in vivo* is affected in Cdc42KO B cells. CCL19, CCL21, CXCL12 and CXCL13 are important chemotactic factors for migration of cells to and within the spleen. Others and we have seen normal migration towards CXCL12 and CXCL13 *in vitro*, this could be translated *in vivo*, even if homing capacity *in vivo* is more complex and also requires expression and function of adhesion molecules. The draining popliteal lymph node accumulates antigens injected in the footpad and thereby potentially attracts more antigen-specific cells independently of Cdc42-expression; this could be another reason for different homing results between the articles. The third reason that may explain the difference in homing capacity of the Cdc42KO B cells is the presence or absence of a specific BCR. As mentioned above, Burbage et al. have crossed $Cdc42^{fl/fl}Mb1^{+/Cre}$ mice with mice expressing BCRs with the high affinity for HEL[224]. This gives the adoptive transferred B cells selective advantage for the HEL antigen in a wild type

host, compared to our model where no additional antigen was injected after transfer. Burbage et al. show that the $Cdc42^{fl/fl}Mb1^{+/Cre}$ B cells have alterations in development of $Cdc42^{fl/fl}Mb1^{+/Cre}$ B cells, which may make it difficult to distinguish the role of Cdc42 in mature cells. To avoid the issue of additive defects from B cell development of Cdc42KO B cells we have taken the approach to use inducible deletion of Cdc42 in mature B cells. By this method we sustain normal B cell development and colonization of the spleen.

2.3.6 The humoral response to foreign antigens

In **paper I-III** we used both TI and TD antigen to evoke immune responses. In **paper II** we used TNP-Ficoll as a TI antigen, to investigate the uptake by MZ B cells and the humoral response 7 days later. As discussed above, an impaired switched humoral response was observed. In **paper III** a TI type 1 immune response was simulated by *in vitro* stimulation of $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ B cells with LPS. This stimulation led to an increase of IgG2b-switched B cells, while switching to IgG3 was similar to that in wild type B cells. This is in line with data from Guo et al., where they show reduced TNP-specific IgM and IgG3 response *in vivo* 7 days after TNP-Ficoll injection of $Cdc42^{fl/fl}CD19^{+/Cre}$ B cells[187], also similar to the reduction found in WKO and cDKO mice in **paper II**.

In **paper I-IV**, we administered *in vivo* the TD antigens TNP-SRBC and NP/TNP-KLH, while anti-CD40 in combination with IL-4 was used to mimic T cell help for *in vitro* assays. Injection of SRBC in mice gives a strong GC reaction, which we also detected in WKO and cDKO mice (data not shown). In **paper I**, TNP was conjugated to SRBC to analyze clonal selection of B cells in WKO and cDKO mice. No clonal expansion was detected (data not shown), most probably because the antigen itself carries too many epitopes. In **paper III**, we investigated the role of Cdc42 in GC B cells after SRBC injection. Since we know that development of Cdc42KO B cells is altered compared to wild type B cells we took advantage of the inducible $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ mice, where we could delete Cdc42 in mature cells, as described above. Both the number of class switched plasma cells and TNP-specific antibody titers were similar in $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ mice and wild type mice. These antibodies may originate from an extrafollicular response since the GC reaction was smaller and $IgG1^{+}$ cells were easily detected in the red pulp and not in B cell follicles in the spleen of $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ mice. In contrast, $Cdc42^{fl/fl}Mb1^{+/Cre}$ mice had no detectable extrafollicular response and reduced GC formation after influenza A virus immunization[188]. I speculate that the clear difference between these experiments mainly derives from that the inducible $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ mice have normal B cell development prior to tamoxifen treatment. The B cell follicles are thereby already populated with mature B cells that, at site, are made deficient of Cdc42. Using the latter model, the function of Cdc42 in B cells during GC formation and immune response can be more accurate investigated.

In vitro stimulation with anti-CD40 and IL-4 showed similar switching to IgG1 and IgE by $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ B cells and wild type B cells. In another *in vivo* experiment both $Cdc42^{fl/fl}CD23^{+/Cre}$ and $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ mice had reduced specific humoral response against NP-KLH, even after boost several weeks after the primary injection. These data with NP-KLH are in line with what both Guo et al. and Burbage et al. present and are in contrast to the normal response we see after SRBC injection or after *in vitro* stimulation. This difference could be due to the potency of SRBC to induce robust immune responses as well as the dose administrated. The importance of dose-dependent response was explained in the introduction with LTα-deficient mice and NP-OVA as antigen for affinity maturation experiments[92].

Westerberg et al. have previously shown that WKO mice are able to form GC upon SRBC immunization, although the response is lower than in wild type mice[174]. The TD antigen response in WKO and cDKO mice was investigated in **paper I** and **II** by immunizations with either NP- or TNP-KLH. The mice were intraperitoneally injected with TNP-KLH in alum, injected a second time three weeks later and sacrificed one week after the boost. The cDKO mice had reduced GC formation at day 28 compared to wild type and WKO mice, quantified both with flow cytometry and immunohistochemistry. The GCs in cDKO mice had no clear polarization (data not shown), detected by light zoned/dark zone B cell markers by flow cytometry as described in Victora et al. 2010. TNP-specific antibody titers were measured weekly during the experiment. Naïve wild type mice have low TNP-specific reactivity and show increase of both IgM and IgG1 antibodies after immunization. In contrast, naïve cDKO mice have significant increased titers of hapten-specific IgM antibodies in serum compared to wild type mice. TNP-specific IgM titers in cDKO mice are similar to that of wild type and WKO mice's response during the experiment, while the IgG1 response is reduced until the boost. Even if less GC B cells were detected at day 28 in cDKO mice, the IgG1 titers were similar as in wild type mice at day 28. This normal antibody production after boost in cDKO mice in **paper II** was not observed in **paper I** (data not shown). This difference may be explained by changes in the experimental setup. In **paper II**, the boost also contained the adjuvant alum, which was not the case in **paper I**. Importantly, all secreted antibodies with any reactivity against TNP were detected in **paper II**, regardless of high or low affinity, while we in **paper I** could distinguish between high- and low-affinity secreted antibodies by using low- or high-coupled NP in the ELISA experiment. This enabled us to detect an increased ratio of high-affinity over low-affinity IgG1 antibodies produced in wild type and WKO mice after boost, which was absent in cDKO mice.

Since the humoral response was reduced in WKO, cDKO and Cdc42KO mice, we wondered weather the cognate T-B cell interaction gave sufficient help for the B cells. B cells from wild type, WKO, cDKO and $Cdc42^{fl/fl}CD23^{+/Cre}$ mice were therefore pre-activated with IL-4 and anti-CD40 for two days followed by OVA-coupling to the BCR and co-cultured with enriched $CD4^+$ T cells from OVA-peptide-specific TCR-transgenic OT-II mice. Three days after co-culture we measured cytokine production and proliferation of the OVA-specific T cells. In **paper I**, neither polarization of B cells, T cell proliferation nor IL-2 production was

different in WKO and cDKO B cell when compared to wild type B cells. In **paper III**, $Cdc42^{fl/fl}CD23^{+/Cre}$ B cells showed a reduced uptake of OVA, which may have contributed to the reduced T cell number producing IL-2 and increased T cell number producing IFNγ compared to co-cultures with wild type B cells. Interestingly, production of IFNγ is associated with a Th1 response, upregulation of MHC molecules and B cells switching to IgG2b[225], which we also see in the $Cdc42^{fl/fl}CD23^{+/Cre}$ B cells *in vitro*. B cells with induced $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ showed similar results to $Cdc42^{fl/fl}CD23^{+/Cre}$ B cells (data not shown). Some of the features we observed in Cdc42KO mice and B cells are also found in SAP-deficient mice, but mainly in T cell-specific SAP-deficiency. In a GC reaction the T_{FH} cells require SAP to make a stable T-B cell interaction, and SAP-deficient mice have impaired memory B cell and plasma cell numbers, while the extrafollicular pathway is less dependent on SAP[226,227]. SAP-deficient mice have increased percentage of $CD4^+$ T cells producing IFNγ, but also IL-2, 30 days after viral infection[119]. There is a possible link between Cdc42-deficiency and SAP-deficiency since SAP interacts with PAK−interacting exchange factor (PIX) upon SLAM receptor activation. PIX is in turn a GEF for Rac1 and Cdc42 and promoting their activation[228,229]. Our findings in **paper III** can be compared to the recent publication of Burbage et al. Their $Cdc42^{fl/fl}Mb1^{+/Cre}$ B cells are as efficient as wild type B cells in BCR internalizing when biotinylated anti-IgM is bound to the BCR, but have a reduction of BCR internalization when antigen is bound, similar to our data in **paper III**. In addition, they found reduced polarization and organization of the antigen inside the $Cdc42^{fl/fl}Mb1^{+/Cre}$ cell[188]. *In vitro* both wild type B cells and $CD4^+$ T cells from OT-II mice have more proliferation than $CD4^+$ T cells and $Cdc42^{fl/fl}Mb1^{+/Cre}$ B cells in co-culture, which is the opposite to what we found. Burbage et al. have also reported a reduction in cognate contact between $Cdc42^{fl/fl}Mb1^{+/Cre}$ B and T cells compared to wild type B cells *in vivo*, by using $Cdc42^{fl/fl}Mb1^{+/Cre}$ HEL-specific B cells, $CD4^+$ T cells from OVA-TCR-transgenic OT-II mice and immunization of HEL-OVA coated microspheres[188]. As Cdc42KO B cells show defect in antigen internalization and intracellular antigen organization, the antigen-presentation on the MHC class II and activation of cognate T cells may also be impaired.

2.3.6.1 Clonal expansion and somatic hypermutations

Since we saw reduced GC formation and antibody specificity towards TNP/NP-KLH but normal B-T cell interactions in WKO and cDKO mice in **paper I** and **II,** we wondered if the clonality of B cells selected in the GCs could a source of the altered response. To investigate clonal expansion we performed spectratyping of cDNA from the CDR3 region of the BCR. CDR1 and CDR2 are in the variable (V) region of the BCR while CDR3 covers the junctions between the V, D and J segments. In **paper I**, mice injected with NP-KLH, showed one clonal expansion distinct from the Gaussian distribution in the V_H1 spectra. This specific peak could represent a monoclonal expansion of cells having the previously described NP-specific high-affinity mutation in position 33 in V_H1-186.2. Since we did not know if WKO

and cDKO B cells have a preferential usage of some of the different V_H families we decided to analyze the most distal which is also the largest family (V_H1), the most proximal (V_H2) and one in mid-distance (V_H5) to the D region. While all B cells from wild type mice mainly used the V_H1 family, variation was seen in WKO and cDKO B cells. To investigate the somatic hypermutations (SHM) of expressed BCRs, we prepared cDNA from GC-enriched B cells and sequenced the productive BCR genes as described in material and methods. The mutation rate was high in IgM and IgG1 BCRs in both wild type and WKO B cells. The NP-specific high-affinity mutation, leading to a tryptophan to leucine exchange (W33L), was detected in 65-80% of the IgG1 clones from wild type and WKO B cells. In cDKO B cells the total mutation frequency was decreased in both IgM and IgG1 BCRs. Surprisingly, the majority (75%) of the IgG1 clones had the W33L mutation, while the rest of the gene kept germline configuration. Unfortunately, only a few cDKO mice gave IgG1 clones to sequence. This difficulty in obtaining switched clones from the V_H1-family reflects the reduced amount of NP-specific IgG1 antibodies found in serum and that the absence of preferential usage of the V_H1 family. With the help of improved techniques, such as next generation sequencing (NGS), we will be able to capture potential clonal expansion outside of the normally used V_H1 family, and also to be able to get many more sequences compared to our 21-109 sequences.

In summary, in **paper I** we show that upon TD antigen challenge cDKO B cells have normal interaction with T cells, but reduced formation and polarization of GC, which leads to a reduction of high-affinity antibodies produced. Since we observed cells carrying the high-affinity mutation in the cDKO mice, but a reduced humoral response, I think it would be interesting to investigate the production of antibodies from individual cells by enzyme-linked immmunospot (ELISpot). Potentially, B cells lacking both WASp and N-WASp are as efficient as wild type B cells at producing high-affinity antibodies, but cannot mount a sufficient response due to their low starting number.

2.3.6.2 Similarities and differences between the mouse phenotypes

Paper I contributes to the existing knowledge that B cells are able to make high-affinity antibodies even when the GC reaction is deviant. DOCK8-deficient mice can initiate GC formation but not sustain the structure. B cells from these mice can also acquire mutations in the CDR regions but lack high-affinity mutations[137]. CXCR5-deficient mice are able to produce high affinity BCRs without being able to form polarized GCs with a dark zone and a light zone[135]. We show reduced GC formation in cDKO mice, by flow cytometry in **paper II** and by immunohistochemistry in **paper I**, where we also show reduced SHM to foreign antigen in cDKO B cells. Nevertheless we do observe the high-affinity mutation. I speculate that this could mean that B cells deficient in both WASp and N-WASp have increased threshold of BCR activation. Since few mutations other than the high-affinity mutation were detected in cDKO B cells, I reason that only the B cells acquiring high affinity during the first

round of proliferation are able to survive. As the wild type and WASp-deficient B cells do not have increased BCR activation threshold they are able to rearrange and test their BCR several times. A potential experiment for investigating the role of BCR signaling in survival and SHM of cDKO GC B cells would be to cross cDKO mice with B1-8 transgenic mice bearing the membrane bound IgM with high-affinity for NP[230]. If it is reduced BCR signaling in cDKO B cells that causes reduced mutation rate GC B cells from cDKO x B1-8 would have more mutations than GC B cells from cDKO mice after NP-KLH immunizations. One reason for increased activation threshold is the reduced LFA-1 expression we detected in cDKO B cells in **paper II**. LFA-1, in interaction with its ligand ICAM-1, reduces the amount of antigen that is needed to bind the BCR for activation, i.e. LFA-1 activation reduces the activating threshold of B cells[231]. Wild type B cells with normal expression of LFA-1 will thus have lower activation threshold than WKO and cDKO B cells. The differences found between WKO and cDKO B cells could be caused by the negative regulation N-WASp has on BCR signaling. We published in collaboration with Dr Song's laboratory that N-WASp suppresses the BCR-mediated activation of B cells, and that Btk-deficient B cells have decreased WASp phosphorylation but increased N-WASp phosphorylation, suggesting different roles in BCR signaling[186]. WKO, cNWKO and cDKO B cells spread on anti-Ig lipid bilayer show that WASp activation promotes actin polymerization and B cell spread in the contact zone, while N-WASp is inhibiting and removing the polymerized actin and makes the synapse structure to contract[186]. These slightly different actions of WASp and N-WASp can be compared to the overall reduction of polymerized actin in Cdc42KO B cells[188]. As mentioned in the introduction, there is a redundancy function between the proteins in actin regulation upon BCR stimulation. WASp, N-WASp and WAVE use the Arp2/3 complex for actin polymerization. WAVE gets activated by the RhoGTPase Rac that may skew the actin polymerization differently than Cdc42 and WASp/N-WASp activation would. It is known that Rac and Cdc42 activation give different phenotype. For example, Rac promotes lamellipodia formation while Cdc42 promotes filopodia formation[194]. Could the reduction of polymerized actin in Cdc42KO B cells be caused by a preferential Rac-mediated activation of WAVE? To elucidate this, Rac activation and phosphorylated WAVE, WASp and N-WASp in Cdc42KO B cells could be measured. It would be interesting to investigate the similarities and differences in phenotype of mice lacking WASp and N-WASp conditionally in B cells and Cdc42-conditional knock out mice, using the same promoter for Cre.

There are most likely several reasons for why some of our data in **paper III** differ from data presented by Guo et al. and Burbage et al. According to Burbage et al. the reason for the striking difference between their study and Guo et al.[187] from 2009 is that there is an incomplete deletion of Cdc42 in B cells from $Cdc42^{fl/fl}CD19^{+/Cre}$ mice compared to their $Cdc42^{fl/fl}Mb1^{+/Cre}$ mice. Even though there might be an incomplete deletion, even in our tamoxifen-induced Cdc42 deletion model, I believe that the major underlying cause of the differences detected is that the B cells in our model are present in the follicle at the moment of Cdc42 deletion. We show that after five constitutive days of tamoxifen treatment 90% of the protein is no longer present. The deletion of Cdc42 in the $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ mouse is

transient and generation of Cre recombinase only occurs as long as tamoxifen, with a half-life in serum of 12 hours, is present[232]. The B cell turnover rate is relatively high in young mice, and in a few days the peripheral B cell pool can be repopulated[233]. The turnover rate in B cells decreases with age, which could influence the result of inducible deletion of Cdc42, if mice of different ages have been used.

In our experiments we have used tamoxifen-treated wild type mice that do not express $Mb1^{+/Cre\text{-}ERT2}$. Tamoxifen is an antagonist for the estrogen receptor ERT2, which blocks B cell maturation by blocking the natural function of estrogen[234]. Estrogen administration leads to BAFF production resulting in an increase in T2 B cells and expansion of autoreactive MZ B cells, which have been associated with lupus disease models in mouse[235,236]. We therefore need to make sure that the defects we see in mature B cell development and GC formation are not caused by the tamoxifen itself but by the Cdc42 deletion. We have reduced the risk for this readout mistake by treating our wild type mice with tamoxifen as well. We have not seen any reduction of B cell numbers in wild type mice treated with tamoxifen compared with untreated mice. Measuring of BAFF titers in wild type and $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ mice before, during and after tamoxifen treatment could tell us if tamoxifen is causing a reduction in B cell populations by lowering systemic BAFF levels. Potentially there could be an increased BAFF production when tamoxifen as an estrogen antagonist is removed.

We have not investigated the B cell maturation in bone marrow in our inducible $Cdc42^{fl/fl}Mb1^{+/Cre\text{-}ERT2}$ mouse but the reduction of transitional B cells in spleen is similar to what Burbage et al. see in their $Cdc42^{fl/fl}Mb1^{+/Cre}$ mouse. The differences we see in antibody titers and plasma cell formation could be due to incomplete deletion of the protein, antigen of choice and the time point at which Cdc42 is deleted in the B cells. As Cdc42 is important for early development, faulty development and selection at early stages could influence the defects we detect in mature cells. I therefore believe it is important to use inducible deletion of the protein when studying its relevance in mature cells.

2.3.7 Increased autoreactive milieu in WASp-deficient mice

Since WAS patients have increased risk of autoimmunity, we were interested in the immune response to autoantigens in our WASp-deficient models (**paper I**). As young WKO and cDKO mice do not spontaneously develop autoimmune-like symptoms, we wanted to induce autoimmunity in a relatively short time frame. In 1998, Elkon's laboratory published that four weekly injection of syngeneic apoptotic thymocytes leads to break of tolerance against autoantigens[237]. Dr Karlsson's laboratory developed this model further by using the synthetic glucocorticoid hormone dexamethasone as an apoptosis inducer[238]. In **paper I** we found that apoptotic cells were captured in the marginal zone of the spleen of WT, WKO and cDKO mice, and all three strains produced DNA-specific IgG antibodies following four injections. Apoptotic cells harbor many self-epitopes, similar to the foreign antigen SRBC. To be able to

screen large spectra of autoreactive antibodies in serum we used an autoantibody array of 95 known autoantigens. Wild type mice showed no autoreactive IgM or IgG before immunizations with apoptotic cells. After antigen challenge wild type serum had increased titers of autoreactive antibodies, mainly of the IgG subclass. Both WKO and cDKO mice were able to mount an autoreactive IgG response after immunization, similar as of wild type mice. Pre-immune WKO mice had increased levels of autoreactive IgG antibodies, as previously shown[183,203]. Interestingly, we also detected increased titers of autoantibodies in naïve cDKO serum, but of IgM subclass. I would like to compare this to the response we got in cDKO mice after injection with the TD antigen NP-KLH. As mentioned earlier, cDKO B cells may require BCRs with high affinity to be able to survive after encountering a foreign antigen, while WKO B cells may mutate their BCR several times before being selected. The same situation might apply here, and the increased threshold for activation may protect the cDKO mice by preventing switching to potential autoreactive and pathogenic IgG, as seen in WKO mice. In a first attempt to investigate clonal expansion after apoptotic cell injection we performed spectratyping on GC-enriched B cells. Unfortunately no clonal expansion could be found (data not shown), most probably due to the numerous epitopes on apoptotic cells. It would be interesting to immunize mice with NP coupled to an autoantigen that has a restricted number of epitopes. Potential autoantigen could be type II collagen leading to collagen arthritis[239]. After immunization, the occurrence of the high-affinity mutation in the V_H1-186.2 would be investigated. There are specific naturally occurring antibody clones found to have reactivity against self, such as phosphorylcholine (PC)[240]. The main producers of this antibody are the B1a B cells in the peritoneal cavity[241], which are not altered in our WKO and cDKO mice. We have not detected any difference in PC reactivity by ELISA on serum from apoptotic cell injected wild type, WKO and cDKO mice (data not shown).

As we observed for both DNA and the hapten NP/TNP, naïve cDKO mice have increased IgM antibodies compared to wild type mice. In **paper II** we could not detect any differences of B1a B cells in the peritoneal cavity of WKO and cDKO mice, while the B1b B cell population is reduced in cDKO. It is previously published that WASp-expressing B1a B cells have a selective advantage in the peritoneal cavity[182,183]. One explanation for the difference in populations is the different markers that have been used to define the B1 B cell populations in peritoneal cavity. B1 B cell numbers are elevated in autoimmune disorders such as SLE, Sjögren's syndrome and rheumatoid arthritis. Of the B1 B cell population the majority is B1a B cells, producing natural polyreactive antibodies with low affinity for several antigens. This polyreactivity gives recognition of autoantigens, which helps in the clearance of apoptotic bodies. Reap et al. stated 1993 that natural antibodies produced by B1 B cells have low pathogenicity and are not the source of the pathogenic autoantibodies in autoimmune disease (SLE)[242]. This statement was contradicted in 2010 when Riemekasten's laboratory published that peritoneal B1a cells are able to undergo CSR to IgG and leave the pleural cavity. They accumulate in the spleen and kidney and become pathogenic both locally and systemically[243]. Since B1a B cells are linked to polyreactive antibodies and autoimmunity it would be of interest to investigate the B1a B cell population in the spleen of both WKO and cDKO mice.

In WKO mice, the autoreactive phenotype is found first in older mice. If the B1 B cells are expanded with age in WKO mice, the differences found in published data compared to our data in **paper II** could be explained by the age difference of mice used. Potentially, the B1 B cells have left the peritoneal cavity in older mice. If we detect elevated B1a B cell number in the spleen of older naïve wild type, WKO and cDKO or after apoptotic cell injection it would be of interest to perform B cell ELISpot targeting PC on B1a purified B cells to investigate the potential autoreactivity of this population.

WKO and especially cDKO mice have reduced antibody titers upon TI (TNP-Ficoll) and reduced GC formation and antibody titers after TD antigen (NP-KLH) immunizations compared to wild type mice. Since we detected antibody production against DNA we were curious to see if WKO and cDKO mice could form GCs to the same extent as wild type mice upon several injections of apoptotic cells. Quantification of GC B cells revealed similar numbers in wild type, WKO and cDKO mice, which is a remarkable difference compared to the response to a foreign antigen. We next wondered if the structure of the GCs in WKO and cDKO mice was similar to that in wild type mice. By staining GC B cells for the specific markers distinguishing light and dark zone B cells, we found that the polarization detected in wild type mice was missing in GCs in WKO and cDKO mice. This could be due to the reduced migration ability of cDKO B cells described in **paper II**, but also to the abnormal localization of follicular dendritic cells (FDCs) discovered in **paper I**, by staining for CD35 in the B cell follicle. In wild type mice, $CD35^+$ cells were mainly located in the GC area towards the marginal zone where the light zone of the GC is located. In WKO and cDKO mice the $CD35^+$ cells covered most parts of the GCs, but also large parts of the B cell follicles. The latter defect in WKO and cDKO mice could actually be beneficial for B cell selection. If the B cells have reduced migratory ability and cannot relocate between dark zone and light zone for proliferation and selection, the unfocused location of FDCs could partly compensate for that problem. Although we could not detect polarization of GCs by localization of $CD35^+$ cells in WKO and cDKO mice by immunohistochemistry, we should explore the presence of specific dark zone GC B cell markers in these mice. There might be preferential areas of B cell proliferation in the GC of WKO and cDKO mice, which is different from wild type mice. For example further away from the FDCs or proliferation everywhere, without specific dark zone formation at all. I favor the latter view because of the broad spread of FDCs and the reduced ability of WKO and cDKO B cells to migrate. Potential markers apart from CXCR4 would be Bcl-6 and AID to detect GC B cells, normally located in the dark zone when polarization occurs[139]. The spread of FDCs could also lead to expansion of autoreactive B cells, since FDCs are a source of BAFF, and BAFF signaling promotes survival of autoreactive B cell clones[61,95]. WKO B cells have normal expression of BAFFR on the cell surface as wild type B cells[183]. Expression of BAFFR on cDKO B cells and BAFF titers in cDKO mice are still unknown.

To be able to detect proliferating cells *in vivo*, we fed the mice with two different DNA incorporating agents 5-ethynyl-2'-deoxyuridine (EdU) and bromodeoxyuridine (BrdU) at different time points, starting with EdU. The advantage of using these thymidine analogues in

drinking water is that they get incorporated into DNA of proliferating cells without influencing the B cell homeostasis. In WKO and cDKO mice we observed GC B cells ($B220^+CD95^+GL7^+$) staying longer in the GC as when compared to wild type GC B cells, while the production of plasma cells was similar in all three strains. In a follow-up experiment, it would be of importance to look into the bone marrow of apoptotic cell injected mice treated with EdU and BrdU. By investigating GC B cells in the spleen we observed that wild type B cells proliferate more and seem to leave the spleen at a higher frequency than WKO and cDKO GC B cells. To verify that the wild type B cells become plasma cells and leave the spleen, and not fall under the limit of detection by diluting their incorporated EdU through proliferating, we need to detect EdU^+ B cells outside the spleen, for example in plasma cells in the bone marrow. For plasma cell migration to the bone marrow CXCL12 and CXCR4 are important[158]. We have detected increased $IgG1^+$ cells in the red pulp of WKO and cDKO mice after apoptotic cell injection. This could be a result of reduced migration capacity towards CXCL12 in the bone marrow, since WKO and cDKO B cells have reduced migratory capacity in *in vitro* assays.

2.4 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

Mutation in amino acid position 112 is one of the most common mutations in AID causing HIGM in human. Our finding of a spontaneous replacement mutation in AID^{R112H} in mice provides a potential and more precise tool than conventional knockout strategies to investigate posttranslational regulation of AID *in vitro* and *in vivo*.

When I got registered as a PhD student, I thought of the question "how can an immune system fail to respond to foreign pathogens while reacting vigorously to autoantigens?", as shown in WAS patients and other primary immunodeficiencies. During my first conference, I got the question slightly differently asked; "how come only 40-70% of the patients develop autoimmunity, and not all of them?". My answer then was that there are different mutations in the *WASp* gene leading to different grades of the disorder. But since then I have thought about it. Now 5 years later, our research may have contributed a small piece to answer this question. Could N-WASp expression in B cells rescue some functions that are reduced because of lack of WASp, as in WAS patients? I would like to investigate the expression of N-WASp in WAS patients. Do patients with more severe WAS and autoimmunity have normal to elevated expression of N-WASp while patients with milder form of WAS have reduced N-WASp expression, which saves them from autoimmune disorders?

We have shown that both WASp and N-WASp have a unique and a partly redundant function in B cells. The selective advantage of B cells expressing the WASp and N-WASp proteins are stronger the further differentiated towards MZ B cells the B cells are. Expression of Cdc42 is more important early in development of B cells than expression of WASp and N-WASp. Potentially WASp and N-WASp, that have been thought to have the same influence on B cells upon Cdc42 activation, actually may skew the B cell response differently. More studies needs to be done in mice where B cells lacking one or a combination of these three proteins.

Our data implies that when MZB cells and MZMs are reduced in number, $CD11c^{+}$ cells occupy the marginal zone area instead. This could have an effect on the selection on which antigens get into the B cell follicle. WKO and cDKO B cells have reduced expression of LFA-1 that may lead to increased BCR activation threshold compared to wild type B cells. The interaction of WKO or cDKO B cells with T helper cells is normal while the localization of antigen presenting FDCs in WKO and cDKO mice is altered. Several of the above mentioned components might be one source for the increased autoreactive milieu in WKO and cDKO mice. We have detected several consequences for the immune system when Cdc42, WASp and/or N-WASp is lacking in B cells. Since there is not one straight interaction pathway from BCR stimulation to actin rearrangement it is difficult to explain all phenotypes found in the different mouse strains. When one signaling pathway is removed, another one might compensate and the phenotype seen is because of overexpression of a compensatory protein instead of reduced expression of the target protein. I would like to continue the research on these proteins on an actin cytoskeleton level and maybe understand

more of the function of these proteins together or compensating for lack of another proteins contributing to the altered immune response in WKO, cDKO and Cdc42KO mice.

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